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Runx2 promotes both osteoblastogenesis and novel osteoclastogenic signals in ST2 mesenchymal progenitor cells

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Abstract

Summary—We profiled the global gene expression of a bone marrow-derived mesenchymal pluripotent cell line in response to Runx2 expression. Besides osteoblast differentiation, Runx2 promoted the osteoclastogenesis of co-cultured splenocytes. This was attributable to the upregulation of many novel osteoclastogenic genes and the downregulation of anti-osteoclastogenic genes.

Introduction—In addition to being a master regulator for osteoblast differentiation, Runx2 controls osteoblast-driven osteoclastogenesis. Previous studies profiling gene expression during osteoblast differentiation had limited focus on Runx2 or paid little attention to its role in mediating osteoblast-driven osteoclastogenesis.

Methods—ST2/Rx2^{dox}, a bone marrow-derived mesenchymal pluripotent cell line that expresses Runx2 in response to Doxycycline (Dox), was used to profile Runx2-induced gene expression changes. Runx2-induced osteoblast differentiation was assessed based on alkaline phosphatase staining and expression of classical marker genes. Osteoclastogenic potential was evaluated by TRAP staining of osteoclasts that differentiated from primary murine splenocytes co-cultured with the ST2/Rx2^{dox} cells. The BeadChip™ platform (Illumina) was used to interrogate genome-wide expression changes in ST2/Rx2^{dox} cultures after treatment with Dox or vehicle for 24 or 48 h. Expression of selected genes was also measured by RT-qPCR.

Results—Dox-mediated Runx2 induction in ST2 cells stimulated their own differentiation along the osteoblast lineage and the differentiation of co-cultured splenocytes into osteoclasts. The latter was attributable to the stimulation of osteoclastogenic genes such as *Sema7a*, *Ltc4s*, *Efnb1*, *Apcdd1*, and *Tnc* as well as the inhibition of anti-osteoclastogenic genes such as *Tnfrsf11b* (OPG), *Sema3a*, *Slco2b1*, *Ogn*, *Clec2d* (*Ocil*), *Il1rn*, and *Rspo2*.

Conclusion—Direct control of osteoblast differentiation and concomitant indirect control of osteoclast differentiation, both through the activity of Runx2 in pre-osteoblasts, constitute a novel mechanism of coordination with a potential crucial role in coupling bone formation and resorption.

Keywords

Co-culture; Microarray; Osteoblast; Osteoclast; Runx2

Introduction

Runx2 is a master transcription factor that controls osteoblast differentiation and bone formation. Runx2 knockout mice completely lack osteoblasts and fail to form mineralized bone [1, 2]. In humans, heterozygous mutation of Runx2 results in the skeletal genetic disease *cleidocranial dysplasia* [3]. Additionally, mice with a hypomorphic Runx2 allele display osteopenia [4], and single nucleotide polymorphism in the human Runx2 locus is associated with variations in bone mineral density [5]. Mice expressing a dominant-negative form of Runx2 in osteoblasts under the control of the osteocalcin promoter are born with a

normal skeleton, but develop a low bone mass phenotype attributable to decreased bone formation [6]. Counterintuitively, transgenic mice expressing a dominant-negative form of Runx2 in cells of the osteoblast lineage under the control of the $\alpha 1(I)$ collagen promoter exhibit high bone mass and are protected from ovariectomy-induced bone loss [7]. Furthermore, mice over-expressing Runx2 under the control of the $\alpha 1(I)$ collagen promoter have low bone mass and develop spontaneous fractures [8, 9]. Taken together these data suggest that Runx2 must be tightly regulated to support skeletal health: Whereas sufficient activity is necessary for adequate osteogenesis, excessive Runx2 activity may lead to osteoporosis [10].

In vitro studies also demonstrate the opposing activities of Runx2. On one hand, inhibition of Runx2 expression impedes osteoblast differentiation [124], and ectopic expression of Runx2 induces the osteoblast phenotype in primary bone marrow stromal cells, pluripotent stem cells, myoblasts, C3H10T1/2 fibroblastic cells, and skin fibroblasts [11–15]. On the other hand, Runx2 overexpression in osteoblasts promotes osteoblast-driven osteoclastogenesis from co-cultured splenocytes [9] or bone marrow osteoclast progenitors [7, 16]. Finally, expression of a dominant-negative Runx2 form in osteoblasts inhibits differentiation of co-cultured osteoclast precursors [7]. Thus, Runx2 appears to play important roles in both osteoblastogenesis and osteoblast-driven osteoclastogenesis, highlighting the importance of its tight regulation.

Despite the importance of Runx2 in skeletal development and bone turnover, and the likely roles of Runx2 in interpreting signals from extracellular matrix proteins [17], TGF β and BMPs [18, 19], Wnts [20], PTH [21], glucocorticoids [22], and sex steroids [10, 23–25], little is known about gene networks regulated by Runx2 in osteoblasts. Most microarray-based gene expression profiling of osteoblast differentiation did not specifically focus on Runx2 [26–30]. Of several Runx2-centered high-throughput gene expression studies, two compared gene expression in skeletal elements of Runx2 knockout versus wild-type embryos [31, 32]. Accompanying the obvious advantages of such in vivo studies are complications related to the tissue heterogeneity and the long history of Runx2 absence since conception of the knockout mice. Other investigators compared the gene expression profiles of C3H 10T1/2 and immortalized mouse calvarial cells possessing or lacking Runx2 [33, 34]. In these studies, Runx2 was severely modified, or cells were analyzed shortly after transfection, which can strongly skew the results. Recently, we and others developed viral vectors facilitating Dox-mediated Runx2 expression in transduced cells [35, 36]. This approach offers synchronous induction of Runx2 expression many days after cells recover from the procedure of introducing the foreign DNA [37]. Here, we investigated changes in gene expression after Dox-mediated induction of Runx2 in bone marrow-derived pluripotent mesenchymal cells. The results are consistent with the known effects of Runx2 on genes related to osteoblast growth and differentiation, but, more importantly, highlight osteoclastogenesis-regulating genes not previously known to reside downstream of Runx2.

Materials and methods

Lentivirus production and infection

The Dox-inducible Runx2 system was previously described [36]. Briefly, Flag-Runx2 cDNA (MASN isoform, type 2) was initially cloned into the SpeI/MfeI-digested lentiviral entry vector pEN_TmiRc3 (ATCC® catalog: MBA-248), and the resulting plasmid was recombined using Gateway® LR Clonase® II enzyme mix (Invitrogen, Carlsbad, CA, USA) with the pSLIK (single lentivector for inducible knockdown) destination vector carrying a hygromycin resistance gene (ATCC® catalog: MBA-237). For packaging, the expression plasmid was co-transfected along with lentiviral helper plasmids pMD.G1 and pCMV R8.91 [38, 39] into HEK293T cells by the calcium chloride method. Culture media containing viral particles were harvested after 48–72 h and used for the transduction of ST2 cells. Transduced ST2 cells (ST2/RX2^{dox}) were then selected by adding Hygromycin B (Invitrogen) to the growth medium at 50 µg/mL. All experimental procedures were carried out at least 2 weeks following Hygromycin B selection.

Cell culture and assays

ST2 and ST2/RX2^{dox} cells were maintained in RPMI-1640 medium supplemented with 10% Tet System Approved FBS from Clontech, CA, USA. Doxycycline (Dox; CalBiochem, La Jolla, CA, USA) was used at 250 ng/mL, unless otherwise stated, and an equal volume of distilled water was used as vehicle control. Cell proliferation was assessed by performing MTT assays (Sigma, St Louis, MO, USA) on the indicated days after seeding of ST2/RX2^{dox} cells at 5,000 cells/well in 24-well plates. To assess the osteoclastogenic potential of the cells, they were plated at 12,000 cells per well in 48-well plates in α MEM supplemented with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific), 10^{-8} M Vit D₃ (Sigma), and 1% penicillin/streptomycin (Gibco). After 1 h, 160,000 primary mouse splenocytes, extracted as described previously [40], were added to each well and the co-cultures were half-fed with Dox or vehicle every other day. On day 13, the cultures were rinsed with phosphate-buffered saline (PBS) and incubated with 1 mg/mL Collagenase P (Roche Applied Sciences, Germany) to remove the ST2/RX2^{dox} cells. Mature osteoclasts were then stained using a TRAP kit (Sigma) and the percentage of TRAP-positive area determined using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Between 1×10^5 and 2×10^5 cells were washed once with PBS and lysed in 200 µL of incubation buffer [100 mM Tris (pH 7.4), 500 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% Nonidet® P-40] supplemented with Complete™ protease inhibitor mix (Roche Diagnostics, Indianapolis, IN, USA). Aliquots of 40 µg cell lysate were subjected to SDS-PAGE, and the proteins were transferred to Amersham Hybond™-P PVDF (GE Healthcare, Piscataway, NJ, USA) membranes and detected with mouse monoclonal antibodies against FLAG® epitope (M2 antibodies from Sigma, Inc.) or against tubulin (developed by Dr. Charles Walsh and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and The University of Iowa, Department of Biological Sciences, Iowa City, USA).

RNA extraction and analysis

Total RNA was isolated using Aurum Total RNA kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's recommendations, and 1 µg was reverse-transcribed using the Superscript III kit (Invitrogen). RT-qPCR was performed using the CFX96™ RT-PCR system, the iQ™ SYBR® Green Supermix (both from Bio-Rad), and the primers listed in Electronic supplementary materials (ESM) Table 1. High-throughput gene expression profiling was performed in biological quadruplicate using the Illumina MouseRef-8 v2.0 expression BeadChip™ platform, which contains 25,697 probes covering 25,600 well-annotated transcripts from over 19,100 unique Mouse genes and 798 negative control probes.

Statistical analysis

Results are expressed as the mean±SD. Each assay was performed in triplicate or quadruplicate and repeated at least twice. Difference between groups was considered significant when $p < 0.05$ using Student's *t* test. The Illumina high-throughput gene expression data were analyzed using the R and Bioconductor packages. The expression values were first subjected to background correction and robust spline normalization after variance-stabilizing transform (VST) using the *lumi* package. The VST transformation takes advantage of a larger number of technical replicates available on Illumina microarrays. Genes differentially expressed after 24 and 48 h of Runx2 induction were identified by fitting a linear model with 2×2 factorial design using the *factDesign* package. Probewise *F* tests were carried out to identify the effect of Runx2 induction on gene expression at 24 and 48 h. Results were considered significant when $p < 0.05$ after Benjamini and Hochberg multiple testing correction using the *multtest* package [41].

Results and discussion

Establishment of ST2 cells conditionally expressing Runx2 (ST2/Rx2^{dox})

ST2 is a murine bone marrow stroma-derived cell line [42] which can differentiate along the adipocytic [43] or osteoblastic lineages [44] and promote the growth and differentiation of early B cells [45], T cells [46], and monocytes/osteoclasts [47]. We engineered an ST2/Rx2^{dox} sub-line which dose-dependently expresses a Flag-tagged Runx2 protein upon treatment with Dox (Fig. 1a). For the present study, we chose the dose of 250 ng/mL Dox, which increased the level of total Runx2 mRNA by 8-fold (Fig. 1b) and resulted in the development of ALP-positive cell nodules (Fig. 1c).

Microarray analysis of Dox-treated ST2/Rx2^{dox} cells confirms stimulation of osteoblast marker genes

We profiled the ST2/Rx2^{dox} cells for their genome-wide gene expression pattern after 1 and 2 days of treatment with either Dox or vehicle in biological quadruplicates (a total of 16 samples). Of 19,100 unique mouse genes interrogated by the microarray, 75 were stimulated by 2.5-fold and 141 were inhibited by 2.5-fold with high statistical significance ($p < 0.001$, ESM Table S2). Unsupervised hierarchical clustering of these 216 genes resulted in a clear separation between the Dox-treated and control samples with little variation among the

biological replicates, demonstrating the overall robustness of the methodology (Fig. 2). In most cases, changes observed after 1 day of treatment were maintained or intensified by day 2. One exception was Runx2 itself. Despite continuous exposure to Dox, Runx2 mRNA levels decreased between days 1 and 2 (Fig. 2), potentially reflecting Runx2-mediated mechanisms responsible for its own mRNA destruction [48, 49]. Among the genes upregulated by Runx2 in ST2 cells were the classical osteoblast markers Osteocalcin/Bglap2, Alkaline phosphatase 2/ALP/Akp2, Osterix/SP7, and Bone Sialoprotein/IBSP (Table 1). The induction of these classical targets, as well as the increased ALP activity (Fig. 1c), indicates that the induced Flag-Runx2 had the overall effect expected from an osteoblast master regulator. Interestingly, the 3- to 18-fold stimulation of these genes was accompanied by an order of magnitude inhibition of the genes encoding Matrix Gla Protein (Mglap) and Mustn1, small proteins (82–84 amino acid residues) highly expressed in non-mineralizing skeletal tissues such as cartilage and tendon [50, 51]. RT-qPCR analysis demonstrated 13-, 27-, and 12-fold stimulation of Bglap, Akp2, and SP7, and 24- and 21-fold inhibition of Mglap and Mustn1, respectively (Fig. 1d).

The microarray results also indicated strong stimulation of the collagen XXIVa1 gene (Table 1), a specific marker of active osteoblasts [52]. As control, Dox treatment of naive ST2 cells affected neither the expression of OC, Sp7, Mglap, or Mustn1 nor the activity of ALP (data not shown).

Runx2 regulates proliferation-related genes in ST2 cells

The probewise linear models of the microarray gene expression data suggested that Runx2 regulated not only biological processes related to bone remodeling and ossification but also those controlling DNA replication and cell division (ESM Fig. S1). Most notably, the data suggested a negative regulation of insulin-like growth factor (IGF) signaling, which plays important roles in promoting osteoblast proliferation [53]. Expression of four genes in the IGF pathway—Igf1, Igf2, Igf2bp3, and Igf2bp2—was inhibited by more than 2.5-fold (Table 2), and Igfbp5 was significantly inhibited by >1.5-fold (ESM Table S2). The inhibition of Igf1 was confirmed by RT-qPCR (Fig. 3a). Consistent with the gene expression data, induction of Runx2 in the Dox-treated cultures inhibited cell proliferation, leading to a 27% reduction in cell accumulation by day 3 (Fig. 3b, c). In addition to repressing IGF-related genes, 48-h Dox treatment stimulated the expression of E2F2 by 2.2-fold (Table 2), potentially contributing to the inhibition of cell proliferation [54].

The inhibition of ST2 cell proliferation by Runx2 (Fig. 3b, c) is consistent with similar results previously obtained with primary calvarial pre-osteoblasts [34, 55], MC3T3-E1 and C2C12 cells [56], as well as prostate cancer cells [36]. However, Runx2 was reported to promote proliferation in other contexts, including in PC3 prostate cancer cells [57] and mammary epithelial cells [58–60]. In fact, our results suggest not only a downregulation of some mitogenic signals but also an upregulation of other mitogenic signals such as Megf10, Calpain 6, and CMM5/CDC46 [61–63] (Table 2). Other changes counteracting the anti-mitogenic effect of Runx2, some of which were also observed in primary osteoblasts [34], include the stimulation of Wnt7b (ESM Table S2) and inhibition of the pro-apoptotic gene Caspase 4 and the tumor suppressor genes Fgl1 [64] and Tspan32 (Table 2). Overall,

however, the inhibition of *Igf1* and other mitogenic genes appears to predominate, resulting in the mild inhibition of ST2 cell proliferation by Runx2 (Fig. 3).

Potential mechanisms of Runx2-mediated osteoclastogenesis

Runx2-deficient mice lack osteoblasts and their skeleton fails to mineralize [1, 2]. Surprisingly, however, over-expression of Runx2 in osteoblasts leads to low bone mass and spontaneous fractures [8, 9, 65]. Furthermore, expression of a dominant-negative (DN) form of Runx2 in osteoblasts results in an increased bone mass and protection from ovariectomy-induced bone loss [7]. Explaining the negative effects of Runx2 on the skeleton at least in part, osteoblasts overexpressing Runx2 promote an exaggerated differentiation of co-cultured pre-osteoclasts [7, 9], and osteoblasts expressing DN-Runx2 fail to induce osteoclast differentiation from pre-osteoclasts [7]. The molecular mechanisms by which Runx2 promotes osteoblast-driven osteoclastogenesis have not been pursued.

We first tested whether Runx2 increased the osteoclastogenic potential of ST2 cells. We cultured ST2/Rx2^{dox} cells with splenocytes and assessed the development of osteoclasts using TRAP staining after 13 days of co-culture with either Dox, to induce Runx2 expression, or vehicle as control. As shown in Fig. 4a, b, Dox-mediated induction of Runx2 in ST2 cells elicited a strong osteoclastogenic response by the co-cultured splenocytes. We therefore screened the microarray dataset for Runx2-mediated changes in gene expression, which could account for the stimulated osteoclastogenesis.

Runx2 upregulates expression of secreted molecules known to promote osteoclastogenesis

Semaphorines: As depicted in Table 3, the microarray data suggested that Dox-mediated stimulation of Runx2 expression resulted in a >4-fold increase in the level of semaphorin 7A (Sema7A) mRNA, and this was confirmed by RT-qPCR (Fig. 4c). In addition to their originally described role in axonal guidance [66], and other roles later reported in vascular growth, tumor progression, and immune response (reviewed in [67]), semaphorins are also implicated in the regulation of bone metabolism. Members of the semaphorin family and their receptors are expressed in cells of the osteoblast and osteoclast lineages, respectively [68–71]. Expression of Sema7a in osteoblasts increases cell migration and stimulates osteoclast fusion [69]. In an in vitro model of periodontal ligament remodeling in tension, where bone formation is stimulated and osteoclastic resorption is inhibited, Sema7A was repressed, consistent with a resorptive role for this semaphorin [71]. Furthermore, human *Sema7A* single nucleotide polymorphisms are associated with decreased bone mass and increased fracture risk [72]. Thus, Runx2-mediated increase in Sema7A in mesenchymal progenitors and osteoblasts likely contributes to their increased ability to induce osteoclastogenesis.

In contrast to Sema7A, Sema3A expression was decreased in response to Runx2 (Table 3). *Sema3A*-null mice exhibit abnormal skeletal development, vertebral fusions, and partial rib duplications [73]. Sema3a expression was also negatively correlated with intervertebral disc resorption and degeneration [74]. Sema3A and 3F together inhibit cell proliferation and affect surrounding endothelial cells by repelling them and inducing their apoptosis [75].

Binding of Sema3A to its receptor Plexin sensitizes cells to Fas-mediated apoptosis by promoting Fas trans-location to lipid raft microdomains before binding with FasL [76]. In the in vitro model of periodontal ligament remodeling in tension mentioned above, the decreased expression of Sema7A was accompanied by an increase in Sema3A and Sema6B expression [71]. In a reciprocal manner, the Runx2-mediated increased expression of Sema7A in our study was accompanied by a decreased expression of Sema3A and Sema6B, as well as Sema3F mRNA (Table 3), consistent with their involvement in Runx2-driven osteoclastogenesis.

Leukotrienes: The microarray analysis of the ST2/Rx2^{dox} cultures suggested that Runx2 increased the mRNA for Leukotriene C4 synthase (*Ltc4s*) by 7-fold (Table 3), and RT-qPCR analysis indicated an even greater, 32-fold, stimulation (Fig. 4c). Leukotrienes are important pro-inflammatory molecules with established and evolving roles in a wide variety of inflammatory diseases (reviewed in [77]). A role for leukotrienes in bone metabolism is strongly suggested by the increased cortical bone thickness and the partial skeletal resistance to ovariectomy observed in mice deficient for 5-Lipoxygenase (5-LO), the enzyme that catalyzes the first step in leukotriene synthesis from arachidonic acid [78]. Furthermore, 5-LO inhibitors attenuate osteoclast differentiation and bone resorption in vitro [79, 80]. The product of the reaction catalyzed by 5-LO, Leukotriene A4 (LTA4), is either hydrolyzed to yield LTB4, which is involved in immune cells chemotaxis, or is conjugated with reduced glutathione to yield Leukotriene C4 (LTC4), a reaction catalyzed by LTC4 Synthase (*Ltc4s*) [81]. LTC4 strongly stimulates osteoclastogenesis and bone resorption in vitro via binding to its monocytic/pre-osteoclast receptor CysLT1 [82, 83]. It also stimulates the expression of IL-11, an osteoclastogenic cytokine [84]. The suggested Runx2-driven leukotriene signaling emanating from ST2 cells may be further amplified by the observed 6.3-fold downregulation of *Slco2b1* (Table 3), encoding a solute carrier organic anion transporter that eliminates leukotrienes and other organic anions [85]. The Dox-mediated upregulation of *Ltc4s* and downregulation of a leukotriene-inactivating gene highlight a potential role for LTC4 in mediating Runx2-regulated osteoblast-driven osteoclastogenesis.

Tenascin C: *Tenascin C* a matrix metalloproteinase [86], binds fibronectin and blocks its interaction with Syndecans. It is expressed in developing bones and increases mineralization in vitro [86]. In a model of fracture healing, in addition to its effect on mineralization, overexpression of Tenascin C also accelerated osteoclast recruitment [86]. Our microarray (Table 3) and RT-qPCR results (Fig. 4c) indicate a dramatic 4- to 6-fold increase in Tenascin C mRNA in response to Runx2, which possibly contributes to the augmented osteoclastogenic activity of the Dox-treated ST2/Rx2^{dox} cells.

Runx2 downregulates expression of secreted molecules known to inhibit osteoclastogenesis—In addition to upregulating mRNAs encoding pro-osteoclastic secreted molecules, Runx2 also decreased expression of the mRNAs for proteins with opposite effects. We already mentioned the downregulation of the semaphorins 3A, 6B, and 3F. Additional inhibitory effects of Runx2 with potential importance to the observed increase in osteoclastogenesis are described below.

Osteoprotegerin: Induction of Runx2 by Dox resulted in decreased osteoprotegerin (OPG) expression (Table 3 and Fig. 4d). Functioning as a decoy receptor for RANKL, a pivotal activator of osteoclastogenesis, a decrease in OPG level is expected to facilitate RANKL activity and thus promote osteoclastogenesis [87, 88]. The decrease in OPG expression, without a change in RANKL expression (ESM Fig. S2), may contribute to the Runx2-mediated osteoclastogenesis in the splenocytes-ST2/Rx2^{dox} co-cultures.

The regulation of OPG expression by Runx2 is ambiguous. Early in vitro studies showed that the promoter region of OPG is positively regulated by Runx2, and in knockout experiments, loss of Runx2 gene dosage results in decreased expression of OPG [89, 90]. However, here and in a previous in vivo study [9], OPG transcription was repressed in Runx2-overexpressing osteoblastic cells. Furthermore, OPG, a target for canonical Wnt signaling [91], may also be indirectly inhibited through the downregulation of the Wnt pathway. That Runx2 inhibits Wnt signaling in ST2 cells is suggested by the stimulation of *Apcdd1*, a Wnt inhibitor [92], and the inhibition of *Rspo2*, both a target and an activator of Wnt signaling [93, 94] (Table 3). The importance of the decrease in *Rspo2* mRNA is underscored by its critical role for Wnt activation specifically in osteoblasts [94]. Plausibly, the inhibition of *Rspo2* and the stimulation of *Apcdd1* in Dox-treated ST2/Rx2^{dox}, through the inhibition of Wnt signaling, contribute to the observed decrease in OPG mRNA and, thus, the increased osteoclastogenesis in the ST2 splenocyte co-cultures (Fig. 4).

Osteoglycin: *Osteoglycin* (IL) (*Ogn*), also known as Mimecan and osteoinductive factor (OIF), is an extracellular protein member of the SLPRS family of proteoglycans and is associated with osteogenesis [95, 96]. Additionally, *Ogn* inhibits osteoclastogenesis [96]. The 5-fold downregulation of *Ogn* expression after 2 days of Dox treatment (Table 3 and Fig. 4d) may therefore contribute to Runx2-induced osteoclastogenesis.

Interleukin 1 receptor antagonist: *Interleukin 1 receptor antagonist* (*Il1rn*) Interleukin-1 (IL1) promotes osteoclastogenesis by activating IL1 receptors on both osteoblasts and osteoclasts [97–99]. Working against IL1, *Il1rn* has been shown to inhibit OVX-induced osteoclastogenesis [100]. Furthermore, a polymorphism in *Il1rn* is associated with increased risks for osteoporotic fractures [101] and periprosthetic osteolysis [102]. Thus, the >5-fold decrease in *Il1rn* mRNA after 48 h of Dox treatment (Table 3 and Fig. 4d) may also contribute to the observed stimulation of osteoclastogenesis.

Runx2-mediated changes in mRNAs encoding membrane-bound osteoclastogenic proteins

Natriuretic peptide receptor 3: *Natriuretic peptide receptor 3* (*Npr3*), known for its role in regulating renal salt excretion and blood pressure, has also been implicated in attenuating osteoclastogenesis and bone turnover [103]. Therefore, the downregulation of *Npr3* mRNA expression (Table 3 and Fig. 4e) potentially contributes to the observed Runx2-mediated osteoclastogenesis in our co-cultures.

C-type lectin domain family 2d: *C-type lectin domain family 2d* (*Clec2d*), a.k.a. osteoclast inhibitory lectin (OCIL), was one of the genes most strongly downregulated by Runx2 in

ST2 cells, and the 5- to 6-fold inhibition suggested by the microarray data (ESM Table S2 and Table 3) was confirmed by RT-qPCR (Fig. 4e). OCIL expressed on the osteoblast membrane strongly inhibits mouse and human osteoclast differentiation [104, 105], and *Ocil* knockout mice suffer increased bone resorption [106]. Thus, inhibition of *Ocil* expression may contribute to the observed Runx2-stimulated osteoclastogenesis.

Ephrin B1: Bidirectional *Eph/Ephrin* signaling has been recently implicated in osteoblast–osteoclast communication (reviewed by [107]). Although such communication has been shown primarily for osteoclastic EphrinB4 and osteoblastic EphB2, involvement of other ephrins and eph receptors is plausible. Runx2 expression in ST2 cells resulted in a >2-fold increase in EphrinB1 (*Efnb1*) mRNA (Table 3 and Fig. 4e). Given the skeletal effects associated with EphB1 deficiency [108, 109] and the decreased peak bone mass and bone size observed in mice lacking *Efnb1* signaling in osteoblasts [110], it is possible that Runx2-mediated *Efnb1* expression contributes to osteoclastogenesis by binding to monocytic EphB receptors [111].

Potential compensatory effects on osteoclastogenesis—We described above several molecular mechanisms potentially mounting the osteoclastogenic response to Runx2. It should be noted, however, that in ST2 cells, Runx2 also elicited reactions that likely keep the osteoclastogenic response in check.

Chemokines: Several mRNAs encoding chemokines with known osteoclastogenic activity were downregulated by ~4-fold in Dox-treated cells (Table 3). These included *Ccl9* and *Cxcl1*, 2, and 12, which are positive regulators of osteoclast differentiation, migration, and bone resorption [112–117].

Notch signaling: Our data also suggest that Runx2 stimulated the anti-osteoclastogenic Notch signaling pathway [118, 119]. Evidence for this is the significant upregulation of the notch target *cdk5r1* and the slight increase in *Hey1* [120, 121] (Table 3 and Fig. 4f). Interestingly, the primary messenger of the Notch pathway, the Notch intracellular domain (NICD), inhibits Runx2 [119], and so do the NICD targets *Hey1* and *Hes1* [122]. In fact, the reported anti-osteoclastogenic and thus bone-sparing property of the Notch pathway [118, 119] may be mediated in part by limiting the activity of Runx2, as do sex steroids [10]. In this sense, our results suggest that Runx2, while promoting the expression of osteoclastogenic genes, also elicits a simple negative feedback loop by activating Notch signaling. Such a feedback loop would subject Runx2 activity and Runx2-mediated osteoclastogenesis to a self-limiting mechanism (Fig. 5).

Runx2-mediated stimulation of other membrane and membrane trafficking-related genes

The Runx2-regulated genes and pathways described so far have been previously implicated in osteoblast growth and differentiation or in osteoclastogenesis. Many of them encode secreted and membrane-associated proteins. Additional mRNAs for membrane-associated proteins, without an established function in bone, were among the top Runx2-regulated genes, and future work is needed to investigate their potential function in bone formation and/or resorption. For example, the strongest response to Runx2 in the microarray dataset

was a 20-fold and a 27-fold stimulation of Tmem114 on days 1 and 2, respectively (ESM Table S2). TMEM114 is a small integral membrane glycoprotein with similarity to eye lens-specific membrane protein 20, epithelial membrane protein 1 (EMP-1), EMP-2, EMP-3, peripheral myelin protein 22 (PMP22), as well as claudin proteins (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=TMEM114>). Following Tmem114 on the list of Runx2-responsive genes were six genes not directly related to membrane trafficking (bglap, Hey1, Ltc4s, Tnc, polr3k, and Akp2), but the next two genes, each responding to Runx2 with a 5-fold increased expression on day 2, were related to membrane trafficking: lysosomal-associated protein transmembrane 5 (Laptm5) and receptor-associated protein of the synapse (Rapsn; ESM Table S2).

Other Runx2-responsive genes related to membrane trafficking include sortilin-related VPS10 domain containing receptor 2 (Sorcs2, 3-fold), the Ca²⁺ transporting ATPase Atp2a3 (3-fold), and synaptosomal-associated protein 47 (Snap47; 3-fold; ESM Table S2). Runx2 also inhibited Caveolin (Cav1, Table 3), whose depletion has been associated with increased exocytosis [123]. That multiple membrane trafficking-related genes are regulated by Runx2 is the basis for current experiments designed to test whether Runx2 controls the movement of osteoclastogenic factors to the plasma membrane for presentation or secretion. These include the canonical osteoclastogenic factors M-CSF and RANKL, as well as non-canonical Runx2-regulated factors such as Sema7A, Ltc4s, and Tenascin C.

In summary, we engineered and profiled gene expression in ST2/Rx2^{dox} cells after induction of Runx2 by Dox. The microarray results, confirmed by RT-qPCR analysis of selected genes, suggest myriad molecular mechanisms underlying Runx2-driven phenotypes of these mesenchymal pluripotent cells, including differentiation toward osteoblasts, modest inhibition of cell proliferation, and, most interestingly, development of a strong capacity to induce osteoclastogenesis in a paracrine manner (Fig. 5). Because sex steroids inhibit Runx2 activity [24, 25], they likely attenuate bone turnover in part by counteracting some of the Runx2-mediated osteoclastogenic mechanisms suggested by the microarray data. The assignment of both osteoblast differentiation [1, 2] and osteoblast-driven osteoclastogenesis [7–9] to Runx2 likely contributes to the coupling of bone formation and resorption.

Supplementary Material

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References

1. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of

- Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 1997; 89:755–764. [PubMed: 9182763]
2. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 1997; 89:765–771. [PubMed: 9182764]
 3. Zhang YW, Yasui N, Kakazu N, Abe T, Takada K, Imai S, Sato M, Nomura S, Ochi T, Okuzumi S, Nogami H, Nagai T, Ohashi H, Ito Y. PEBP2alphaA/CBFA1 mutations in Japanese cleidocranial dysplasia patients. *Gene*. 2000; 244:21–28. [PubMed: 10689183]
 4. Xiao Z, Awad HA, Liu S, Mahlios J, Zhang S, Guilak F, Mayo MS, Quarles LD. Selective Runx2-II deficiency leads to low-turnover osteopenia in adult mice. *Dev Biol*. 2005; 283:345–356. [PubMed: 15936013]
 5. Vaughan T, Pasco JA, Kotowicz MA, Nicholson GC, Morrison NA. Alleles of RUNX2/CBFA1 gene are associated with differences in bone mineral density and risk of fracture. *J Bone Miner Res*. 2002; 17:1527–1534. [PubMed: 12162506]
 6. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev*. 1999; 13:1025–1036. [PubMed: 10215629]
 7. Maruyama Z, Yoshida CA, Furuichi T, Amizuka N, Ito M, Fukuyama R, Miyazaki T, Kitaura H, Nakamura K, Fujita T, Kanatani N, Moriishi T, Yamana K, Liu W, Kawaguchi H, Nakamura K, Komori T. Runx2 determines bone maturity and turnover rate in postnatal bone development and is involved in bone loss in estrogen deficiency. *Dev Dyn*. 2007; 236:1876–1890. [PubMed: 17497678]
 8. Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A, Komori T. Over-expression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J Cell Biol*. 2001; 155:157–166. [PubMed: 11581292]
 9. Geoffroy V, Kneissel M, Fournier B, Boyde A, Matthias P. High bone resorption in adult aging transgenic mice over-expressing cbfa1/runx2 in cells of the osteoblastic lineage. *Mol Cell Biol*. 2002; 22:6222–6233. [PubMed: 12167715]
 10. Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O, Gabet Y. Regulation of adult bone turnover by sex steroids. *J Cell Physiol*. 2010; 224:305–310. [PubMed: 20432458]
 11. Byers BA, Garcia AJ. Exogenous Runx2 expression enhances in vitro osteoblastic differentiation and mineralization in primary bone marrow stromal cells. *Tissue Eng*. 2004; 10:1623–1632. [PubMed: 15684671]
 12. Tashiro K, Inamura M, Kawabata K, Sakurai F, Yamanishi K, Hayakawa T, Mizuguchi H. Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction. *Stem Cells*. 2009; 27:1802–1811. [PubMed: 19544436]
 13. Gersbach CA, Guldberg RE, Garcia AJ. In vitro and in vivo osteoblastic differentiation of BMP-2- and Runx2-engineered skeletal myoblasts. *J Cell Biochem*. 2007; 100:1324–1336. [PubMed: 17131362]
 14. Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M, Ogita K, Komori T. Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. *J Cell Biol*. 2004; 166:85–95. [PubMed: 15226309]
 15. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 1997; 89:747–754. [PubMed: 9182762]
 16. Enomoto H, Shiojiri S, Hoshi K, Furuichi T, Fukuyama R, Yoshida CA, Kanatani N, Nakamura R, Mizuno A, Zanma A, Yano K, Yasuda H, Higashio K, Takada K, Komori T. Induction of osteoclast differentiation by Runx2 through receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin regulation and partial rescue of osteoclastogenesis in Runx2^{-/-} mice by RANKL transgene. *J Biol Chem*. 2003; 278:23971–23977. [PubMed: 12697767]
 17. Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. *J Biol Chem*. 2002; 277:36181–36187. [PubMed: 12110689]

18. Alliston T, Choy L, Ducey P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J*. 2001; 20:2254–2272. [PubMed: 11331591]
19. Phimpililai M, Zhao Z, Boules H, Roca H, Franceschi RT. BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *J Bone Miner Res*. 2006; 21:637–646. [PubMed: 16598384]
20. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem*. 2005; 280:33132–33140. [PubMed: 16043491]
21. Hisa I, Inoue Y, Hendy GN, Canaff L, Kitazawa R, Kitazawa S, Komori T, Sugimoto T, Seino S, Kaji H. Parathyroid hormone-responsive Smad3-related factor, Tmem119, promotes osteoblast differentiation and interacts with the bone morphogenetic protein-Runx2 pathway. *J Biol Chem*. 2011; 286:9787–9796. [PubMed: 21239498]
22. Chang DJ, Ji C, Kim KK, Casinghino S, McCarthy TL, Centrella M. Reduction in transforming growth factor beta receptor I expression and transcription factor CBFA1 on bone cells by glucocorticoid. *J Biol Chem*. 1998; 273:4892–4896. [PubMed: 9478931]
23. McCarthy TL, Chang WZ, Liu Y, Centrella M. Runx2 integrates estrogen activity in osteoblasts. *J Biol Chem*. 2003; 278:43121–43129. [PubMed: 12951324]
24. Khalid O, Baniwal SK, Purcell DJ, Leclerc N, Gabet Y, Stallcup MR, Coetzee GA, Frenkel B. Modulation of Runx2 activity by estrogen receptor-alpha: implications for osteoporosis and breast cancer. *Endocrinology*. 2008; 149:5984–5995. [PubMed: 18755791]
25. Baniwal SK, Khalid O, Sir D, Buchanan G, Coetzee GA, Frenkel B. Repression of Runx2 by androgen receptor (AR) in osteoblasts and prostate cancer cells: AR binds Runx2 and abrogates its recruitment to DNA. *Mol Endocrinol*. 2009; 23:1203–1214. [PubMed: 19389811]
26. de Jong DS, van Zoelen EJ, Bauerschmidt S, Olijve W, Steegenga WT. Microarray analysis of bone morphogenetic protein, transforming growth factor beta, and activin early response genes during osteoblastic cell differentiation. *J Bone Miner Res*. 2002; 17:2119–2129. [PubMed: 12469906]
27. Garcia T, Roman-Roman S, Jackson A, Theilhaber J, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R. Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro. *Bone*. 2002; 31:205–211. [PubMed: 12110436]
28. Roman-Roman S, Garcia T, Jackson A, Theilhaber J, Rawadi G, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R. Identification of genes regulated during osteoblastic differentiation by genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro. *Bone*. 2003; 32:474–482. [PubMed: 12753863]
29. Qi H, Aguiar DJ, Williams SM, La Pean A, Pan W, Verfaillie CM. Identification of genes responsible for osteoblast differentiation from human mesodermal progenitor cells. *Proc Natl Acad Sci USA*. 2003; 100:3305–3310. [PubMed: 12631704]
30. Kulterer B, Friedl G, Jandrositz A, Sanchez-Cabo F, Prokesh A, Paar C, Scheideler M, Windhager R, Preisegger KH, Trajanoski Z. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics*. 2007; 8:70. [PubMed: 17352823]
31. Vaes BL, Ducey P, Sijbers AM, Hendriks JM, van Someren EP, de Jong NG, van den Heuvel ER, Olijve W, van Zoelen EJ, Decherling KJ. Microarray analysis on Runx2-deficient mouse embryos reveals novel Runx2 functions and target genes during intramembranous and endochondral bone formation. *Bone*. 2006; 39:724–738. [PubMed: 16774856]
32. Hecht J, Seitz V, Urban M, Wagner F, Robinson PN, Stiege A, Dieterich C, Kornak U, Wilkening U, Brieske N, Zwingman C, Kidess A, Stricker S, Mundlos S. Detection of novel skeletogenesis target genes by comprehensive analysis of a Runx2(−/−) mouse model. *Gene Expr Patterns*. 2007; 7:102–112. [PubMed: 16829211]
33. Stock M, Schafer H, Fliegau M, Otto F. Identification of novel genes of the bone-specific transcription factor Runx2. *J Bone Miner Res*. 2004; 19:959–972. [PubMed: 15190888]

34. Teplyuk NM, Galindo M, Teplyuk VI, Pratap J, Young DW, Lapointe D, Javed A, Stein JL, Lian JB, Stein GS, van Wijnen AJ. Runx2 regulates G protein-coupled signaling pathways to control growth of osteoblast progenitors. *J Biol Chem*. 2008; 283:27585–27597. [PubMed: 18625716]
35. Gersbach CA, Le Doux JM, Guldberg RE, Garcia AJ. Inducible regulation of Runx2-stimulated osteogenesis. *Gene Ther*. 2006; 13:873–882. [PubMed: 16496016]
36. Baniwal SK, Khalid O, Gabet Y, Shah RR, Purcell DJ, Mav D, Kohn-Gabet AE, Shi Y, Coetzee GA, Frenkel B. Runx2 transcriptome of prostate cancer cells: insights into invasiveness and bone metastasis. *Mol Cancer*. 2010; 9:258. [PubMed: 20863401]
37. Jacobsen L, Calvin S, Lobenhofer E. Transcriptional effects of transfection: the potential for misinterpretation of gene expression data generated from transiently transfected cells. *Biotechniques*. 2009; 47:617–624. [PubMed: 19594446]
38. Phillips JE, Garcia AJ. Retroviral-mediated gene therapy for the differentiation of primary cells into a mineralizing osteoblastic phenotype. *Methods Mol Biol*. 2008; 433:333–354. [PubMed: 18679633]
39. Kim JH, Yang CK, Heo K, Roeder RG, An W, Stallcup MR. CCAR1, a key regulator of mediator complex recruitment to nuclear receptor transcription complexes. *Mol Cell*. 2008; 31:510–519. [PubMed: 18722177]
40. Gabet Y, Baniwal SK, Leclerc N, Shi Y, Kohn-Gabet AE, Cogan J, Dixon A, Bachar M, Guo L, Turman JE Jr, Frenkel B. Krox20/EGR2 deficiency accelerates cell growth and differentiation in the monocytic lineage and decreases bone mass. *Blood*. 2010; 116:3964–3971. [PubMed: 20716776]
41. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*. 1995; 57:289–300.
42. Ogawa M, Nishikawa S, Ikuta K, Yamamura F, Naito M, Takahashi K, Nishikawa S. B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *EMBO J*. 1988; 7:1337–1343. [PubMed: 3261687]
43. Ding J, Nagai K, Woo JT. Insulin-dependent adipogenesis in stromal ST2 cells derived from murine bone marrow. *Biosci Biotechnol Biochem*. 2003; 67:314–321. [PubMed: 12728992]
44. Otsuka E, Yamaguchi A, Hirose S, Hagiwara H. Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am J Physiol*. 1999; 277:C132–138. [PubMed: 10409116]
45. Hardy RR, Kishimoto T, Hayakawa K. Differentiation of B cell progenitors in vitro: generation of surface IgM⁺ B cells, including Ly-1 B cells, from Thy-1[−] asialoGM1⁺ cells in newborn liver. *Eur J Immunol*. 1987; 17:1769–1774. [PubMed: 2891538]
46. Tong J, Kishi H, Matsuda T, Muraguchi A. A bone marrow-derived stroma cell line, ST2, can support the differentiation of fetal thymocytes from the CD4⁺ CD8⁺ double negative to the CD4⁺ CD8⁺ double positive differentiation stage in vitro. *Immunology*. 1999; 97:672–678. [PubMed: 10457222]
47. Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc Natl Acad Sci USA*. 1990; 87:7260–7264. [PubMed: 2169622]
48. Pregizer S, Baniwal SK, Yan X, Borok Z, Frenkel B. Progressive recruitment of Runx2 to genomic targets despite decreasing expression during osteoblast differentiation. *J Cell Biochem*. 2008; 105:965–970. [PubMed: 18821584]
49. Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, van Wijnen AJ, Stein GS. A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proc Natl Acad Sci U S A*. 2011; 108:9863–9868. www.pnas.org/cgi/doi/10.1073/pnas.1018493108. [PubMed: 21628588]
50. Luo G, D'Souza R, Hogue D, Karsenty G. The matrix Gla protein gene is a marker of the chondrogenesis cell lineage during mouse development. *J Bone Miner Res*. 1995; 10:325–334. [PubMed: 7754814]

51. Murshed M, Schinke T, McKee MD, Karsenty G. Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J Cell Biol.* 2004; 165:625–630. [PubMed: 15184399]
52. Matsuo N, Tanaka S, Yoshioka H, Koch M, Gordon MK, Ramirez F. Collagen XXIV (Col24a1) gene expression is a specific marker of osteoblast differentiation and bone formation. *Connect Tissue Res.* 2008; 49:68–75. [PubMed: 18382892]
53. Mohan S, Nakao Y, Honda Y, Landale E, Leser U, Dony C, Lang K, Baylink DJ. Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. *J Biol Chem.* 1995; 270:20424–20431. [PubMed: 7544787]
54. Infante A, Laresgoiti U, Fernandez-Rueda J, Fullaondo A, Galan J, Diaz-Uriarte R, Malumbres M, Field SJ, Zubiaga AM. E2F2 represses cell cycle regulators to maintain quiescence. *Cell Cycle (Georgetown Tex).* 2008; 7:3915–3927.
55. Pratap J, Galindo M, Zaidi SK, Vradii D, Bhat BM, Robinson JA, Choi JY, Komori T, Stein JL, Lian JB, Stein GS, van Wijnen AJ. Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. *Cancer Res.* 2003; 63:5357–5362. [PubMed: 14500368]
56. Galindo M, Pratap J, Young DW, Hovhannisyan H, Im HJ, Choi JY, Lian JB, Stein JL, Stein GS, van Wijnen AJ. The bone-specific expression of Runx2 oscillates during the cell cycle to support a G1-related antiproliferative function in osteoblasts. *J Biol Chem.* 2005; 280:20274–20285. [PubMed: 15781466]
57. van der Deen M, Akech J, Wang T, FitzGerald TJ, Altieri DC, Languino LR, Lian JB, van Wijnen AJ, Stein JL, Stein GS. The cancer-related Runx2 protein enhances cell growth and responses to androgen and TGFbeta in prostate cancer cells. *J Cell Biochem.* 2010; 109:828–837. [PubMed: 20082326]
58. Pratap J, Imbalzano KM, Underwood JM, Cohet N, Gokul K, Akech J, van Wijnen AJ, Stein JL, Imbalzano AN, Nickerson JA, Lian JB, Stein GS. Ectopic runx2 expression in mammary epithelial cells disrupts formation of normal acini structure: implications for breast cancer progression. *Cancer Res.* 2009; 69:6807–6814. [PubMed: 19690135]
59. Qiao M, Shapiro P, Fosbrink M, Rus H, Kumar R, Passaniti A. Cell cycle-dependent phosphorylation of the RUNX2 transcription factor by cdc2 regulates endothelial cell proliferation. *J Biol Chem.* 2006; 281:7118–7128. [PubMed: 16407259]
60. Mikasa M, Rokutanda S, Komori H, Ito K, Tsang YS, Date Y, Yoshida CA, Komori T. Regulation of Tcf7 by Runx2 in chondrocyte maturation and proliferation. *J Bone Miner Metab.* 2011; 29:291–299. [PubMed: 20890621]
61. Holterman CE, Le Grand F, Kuang S, Seale P, Rudnicki MA. Megf10 regulates the progression of the satellite cell myogenic program. *J Cell Biol.* 2007; 179:911–922. [PubMed: 18056409]
62. Liu Y, Mei C, Sun L, Li X, Liu M, Wang L, Li Z, Yin P, Zhao C, Shi Y, Qiu S, Fan J, Zha X. The PI3K-Akt pathway regulates calpain 6 expression, proliferation, and apoptosis. *Cell Signal.* 2011; 23:827–836. [PubMed: 21255642]
63. Hardy CF, Dryga O, Seematter S, Pahl PM, Sclafani RA. mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. *Proc Natl Acad Sci USA.* 1997; 94:3151–3155. [PubMed: 9096361]
64. Yan J, Yu Y, Wang N, Chang Y, Ying H, Liu W, He J, Li S, Jiang W, Li Y, Liu H, Wang H, Xu Y. LFIRE-1/HFRE-1, a liver-specific gene, is frequently downregulated and has growth suppressor activity in hepatocellular carcinoma. *Oncogene.* 2004; 23:1939–1949. [PubMed: 14981537]
65. Kanatani N, Fujita T, Fukuyama R, Liu W, Yoshida CA, Moriishi T, Yamana K, Miyazaki T, Toyosawa S, Komori T. Cbf beta regulates Runx2 function isoform-dependently in postnatal bone development. *Dev Biol.* 2006; 296:48–61. [PubMed: 16797526]
66. Culotti JG, Kolodkin AL. Functions of netrins and semaphorins in axon guidance. *Curr Opin Neurobiol.* 1996; 6:81–88. [PubMed: 8794052]
67. Kikutani H, Suzuki K, Kumanogoh A. Immune semaphorins: increasing members and their diverse roles. *Adv Immunol.* 2007; 93:121–143. [PubMed: 17383540]
68. Lallier TE. Semaphorin profiling of periodontal fibroblasts and osteoblasts. *J Dent Res.* 2004; 83:677–682. [PubMed: 15329371]

69. Delorme G, Saltel F, Bonnelye E, Jurdic P, Machuca-Gayet I. Expression and function of semaphorin 7A in bone cells. *Biology of the Cell/under the auspices of the European Cell Biology Organization*. 2005; 97:589–597.
70. Ji JD, Park-Min KH, Ivashkiv LB. Expression and function of semaphorin 3A and its receptors in human monocyte-derived macrophages. *Hum Immunol*. 2009; 70:211–217. [PubMed: 19480842]
71. Spencer AY, Lallier TE. Mechanical tension alters semaphorin expression in the periodontium. *J Periodontol*. 2009; 80:1665–1673. [PubMed: 19792857]
72. Koh JM, Oh B, Lee JY, Lee JK, Kimm K, Kim GS, Park BL, Cheong HS, Shin HD, Hong JM, Kim TH, Park EK, Kim SY. Association study of semaphorin 7a (sema7a) polymorphisms with bone mineral density and fracture risk in postmenopausal Korean women. *J Hum Genet*. 2006; 51:112–117. [PubMed: 16372136]
73. Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature*. 1996; 383:525–528. [PubMed: 8849723]
74. Tolofari SK, Richardson SM, Freemont AJ, Hoyland JA. Expression of semaphorin 3A and its receptors in the human intervertebral disc: potential role in regulating neural ingrowth in the degenerate intervertebral disc. *Arthritis Res Ther*. 2010; 12:R1. [PubMed: 20051117]
75. Guttmann-Raviv N, Shraga-Heled N, Varshavsky A, Guimaraes-Sternberg C, Kessler O, Neufeld G. Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. *J Biol Chem*. 2007; 282:26294–26305. [PubMed: 17569671]
76. Moretti S, Procopio A, Lazzarini R, Rippo MR, Testa R, Marra M, Tamagnone L, Catalano A. Semaphorin3A signaling controls Fas (CD95)-mediated apoptosis by promoting Fas translocation into lipid rafts. *Blood*. 2008; 111:2290–2299. [PubMed: 18056484]
77. Werz O, Steinhilber D. Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacol Ther*. 2006; 112:701–718. [PubMed: 16837050]
78. Bonewald LF, Flynn M, Qiao M, Dallas MR, Mundy GR, Boyce BF. Mice lacking 5-lipoxygenase have increased cortical bone thickness. *Adv Exp Med Biol*. 1997; 433:299–302. [PubMed: 9561156]
79. Franchi-Miller C, Saffar JL. The 5-lipoxygenase inhibitor BWA4C impairs osteoclastic resorption in a synchronized model of bone remodeling. *Bone*. 1995; 17:185–191. [PubMed: 8554928]
80. Anderson GI, MacQuarrie R, Osinga C, Chen YF, Langman M, Gilbert R. Inhibition of leukotriene function can modulate particulate-induced changes in bone cell differentiation and activity. *J Biomed Mater Res*. 2001; 58:406–414. [PubMed: 11410899]
81. Tantisira KG, Drazen JM. Genetics and pharmacogenetics of the leukotriene pathway. *J Allergy Clin Immunol*. 2009; 124:422–427. [PubMed: 19665766]
82. Gallwitz WE, Mundy GR, Lee CH, Qiao M, Roodman GD, Raftery M, Gaskell SJ, Bonewald LF. 5-Lipoxygenase metabolites of arachidonic acid stimulate isolated osteoclasts to resorb calcified matrices. *J Biol Chem*. 1993; 268:10087–10094. [PubMed: 8486677]
83. Garcia C, Qiao M, Chen D, Kirchen M, Gallwitz W, Mundy GR, Bonewald LF. Effects of synthetic peptido-leukotrienes on bone resorption in vitro. *J Bone Miner Res*. 1996; 11:521–529. [PubMed: 8992883]
84. Lee KS, Kim SR, Park HS, Park SJ, Min KH, Lee KY, Jin SM, Lee YC. Cysteinyl leukotriene upregulates IL-11 expression in allergic airway disease of mice. *J Allergy Clin Immunol*. 2007; 119:141–149. [PubMed: 17208595]
85. Nishio T, Adachi H, Nakagomi R, Tokui T, Sato E, Tanemoto M, Fujiwara K, Okabe M, Onogawa T, Suzuki T, Nakai D, Shiiba K, Suzuki M, Ohtani H, Kondo Y, Unno M, Ito S, Iinuma K, Nunoki K, Matsuno S, Abe T. Molecular identification of a rat novel organic anion transporter moat1, which transports prostaglandin D(2), leukotriene C(4), and taurocholate. *Biochem Biophys Res Commun*. 2000; 275:831–838. [PubMed: 10973807]
86. Cambell, T., Wong, W., Fassler, R., Mackie, E. *Biology of thrombospondins and other modulatory extracellular matrix proteins*. University of Wisconsin; Madison: 2000. Abberant bone repair in tenascin-C knockout mice.
87. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine

- that regulates osteoclast differentiation and activation. *Cell*. 1998; 93:165–176. [PubMed: 9568710]
88. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA*. 1998; 95:3597–3602. [PubMed: 9520411]
 89. Thirunavukkarasu K, Halladay DL, Miles RR, Yang X, Galvin RJ, Chandrasekhar S, Martin TJ, Onyia JE. The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. *J Biol Chem*. 2000; 275:25163–25172. [PubMed: 10833509]
 90. Gao YH, Shinki T, Yuasa T, Kataoka-Enomoto H, Komori T, Suda T, Yamaguchi A. Potential role of cbfa1, an essential transcriptional factor for osteoblast differentiation, in osteoclastogenesis: regulation of mRNA expression of osteoclast differentiation factor (ODF). *Biochem Biophys Res Commun*. 1998; 252:697–702. [PubMed: 9837769]
 91. Glass DA 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA, Karsenty G. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell*. 2005; 8:751–764. [PubMed: 15866165]
 92. Shimomura Y, Agalliu D, Vonica A, Luria V, Wajid M, Baumer A, Belli S, Petukhova L, Schinzel A, Brivanlou AH, Barres BA, Christiano AM. APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. *Nature*. 2010; 464:1043–1047. [PubMed: 20393562]
 93. Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W. R-Spondin2 is a secreted activator of Wnt/beta-catenin signaling and is required for *Xenopus* myogenesis. *Dev Cell*. 2004; 7:525–534. [PubMed: 15469841]
 94. Friedman MS, Oyserman SM, Hankenson KD. Wnt11 promotes osteoblast maturation and mineralization through R-spondin 2. *J Biol Chem*. 2009; 284:14117–14125. [PubMed: 19213727]
 95. Balint E, Lapointe D, Drissi H, van der Meijden C, Young DW, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Phenotype discovery by gene expression profiling: mapping of biological processes linked to BMP-2-mediated osteoblast differentiation. *J Cell Biochem*. 2003; 89:401–426. [PubMed: 12704803]
 96. Kukita A, Bonewald L, Rosen D, Seyedin S, Mundy GR, Roodman GD. Osteoinductive factor inhibits formation of human osteoclast-like cells. *Proc Natl Acad Sci USA*. 1990; 87:3023–3026. [PubMed: 2326263]
 97. Akatsu T, Takahashi N, Udagawa N, Imamura K, Yamaguchi A, Sato K, Nagata N, Suda T. Role of prostaglandins in interleukin-1-induced bone resorption in mice in vitro. *J Bone Miner Res*. 1991; 6:183–189. [PubMed: 2028836]
 98. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone*. 1999; 25:255–259. [PubMed: 10495128]
 99. Lee YM, Fujikado N, Manaka H, Yasuda H, Iwakura Y. IL-1 plays an important role in the bone metabolism under physiological conditions. *Int Immunol*. 2010; 22:805–816. [PubMed: 20679512]
 100. Koh JM, Lee YS, Kim YS, Park SH, Lee SH, Kim HH, Lee MS, Lee KU, Kim GS. Heat shock protein 60 causes osteoclastic bone resorption via Toll-like receptor-2 in estrogen deficiency. *Bone*. 2009; 45:650–660. [PubMed: 19527807]
 101. Langdahl BL, Lokke E, Carstens M, Stenkjaer LL, Eriksen EF. Osteoporotic fractures are associated with an 86-base pair repeat polymorphism in the interleukin-1-receptor antagonist gene but not with polymorphisms in the interleukin-1beta gene. *J Bone Miner Res*. 2000; 15:402–414. [PubMed: 10750554]
 102. Gordon A, Kiss-Toth E, Stockley I, Eastell R, Wilkinson JM. Polymorphisms in the interleukin-1 receptor antagonist and interleukin-6 genes affect risk of osteolysis in patients with total hip arthroplasty. *Arthritis Rheum*. 2008; 58:3157–3165. [PubMed: 18821666]
 103. Matsukawa N, Grzesik WJ, Takahashi N, Pandey KN, Pang S, Yamauchi M, Smithies O. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. *Proc Natl Acad Sci USA*. 1999; 96:7403–7408. [PubMed: 10377427]

104. Zhou H, Kartsogiannis V, Hu YS, Elliott J, Quinn JM, McKinstry WJ, Gillespie MT, Ng KW. A novel osteoblast-derived C-type lectin that inhibits osteoclast formation. *J Biol Chem*. 2001; 276:14916–14923. [PubMed: 11278931]
105. Hu YS, Zhou H, Myers D, Quinn JM, Atkins GJ, Ly C, Gange C, Kartsogiannis V, Elliott J, Kostakis P, Zannettino AC, Cromer B, McKinstry WJ, Findlay DM, Gillespie MT, Ng KW. Isolation of a human homolog of osteoclast inhibitory lectin that inhibits the formation and function of osteoclasts. *J Bone Miner Res*. 2004; 19:89–99. [PubMed: 14753741]
106. Kartsogiannis V, Sims NA, Quinn JM, Ly C, Cipetic M, Poulton IJ, Walker EC, Saleh H, McGregor NE, Wallace ME, Smyth MJ, Martin TJ, Zhou H, Ng KW, Gillespie MT. Osteoclast inhibitory lectin, an immune cell product that is required for normal bone physiology in vivo. *J Biol Chem*. 2008; 283:30850–30860. [PubMed: 18782774]
107. Edwards CM, Mundy GR. Eph receptors and ephrin signaling pathways: a role in bone homeostasis. *Int J Med Sci*. 2008; 5:263–272. [PubMed: 18797510]
108. Compagni A, Logan M, Klein R, Adams RH. Control of skeletal patterning by ephrinB1–EphB interactions. *Dev Cell*. 2003; 5:217–230. [PubMed: 12919674]
109. Davy A, Aubin J, Soriano P. Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev*. 2004; 18:572–583. [PubMed: 15037550]
110. Xing W, Kim J, Wergedal J, Chen ST, Mohan S. Ephrin B1 regulates bone marrow stromal cell differentiation and bone formation by influencing TAZ transactivation via complex formation with NHERF1. *Mol Cell Biol*. 2010; 30:711–721. [PubMed: 19995908]
111. Matsuo K. Eph and ephrin interactions in bone. *Adv Exp Med Biol*. 2010; 658:95–103. [PubMed: 19950019]
112. Yang M, Mailhot G, MacKay CA, Mason-Savas A, Aubin J, Odgren PR. Chemokine and chemokine receptor expression during colony stimulating factor-1-induced osteoclast differentiation in the toothless osteopetrotic rat: a key role for CCL9 (MIP-1gamma) in osteoclastogenesis in vivo and in vitro. *Blood*. 2006; 107:2262–2270. [PubMed: 16304045]
113. Grassi F, Cristino S, Toneguzzi S, Piacentini A, Facchini A, Lisignoli G. CXCL12 chemokine up-regulates bone resorption and MMP-9 release by human osteoclasts: CXCL12 levels are increased in synovial and bone tissue of rheumatoid arthritis patients. *J Cell Physiol*. 2004; 199:244–251. [PubMed: 15040007]
114. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 1996; 382:635–638. [PubMed: 8757135]
115. Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. *Bone*. 2005; 36:840–853. [PubMed: 15794931]
116. Lu Y, Cai Z, Xiao G, Keller ET, Mizokami A, Yao Z, Roodman GD, Zhang J. Monocyte chemotactic protein-1 mediates prostate cancer-induced bone resorption. *Cancer Res*. 2007; 67:3646–3653. [PubMed: 17440076]
117. Onan D, Allan EH, Quinn JM, Gooi JH, Pompolo S, Sims NA, Gillespie MT, Martin TJ. The chemokine Cxcl1 is a novel target gene of parathyroid hormone (PTH)/PTH-related protein in committed osteoblasts. *Endocrinology*. 2009; 150:2244–2253. [PubMed: 19147675]
118. Yamada T, Yamazaki H, Yamane T, Yoshino M, Okuyama H, Tsuneto M, Kurino T, Hayashi S, Sakano S. Regulation of osteoclast development by Notch signaling directed to osteoclast precursors and through stromal cells. *Blood*. 2003; 101:2227–2234. [PubMed: 12411305]
119. Engin F, Yao Z, Yang T, Zhou G, Bertin T, Jiang MM, Chen Y, Wang L, Zheng H, Sutton RE, Boyce BF, Lee B. Dimorphic effects of Notch signaling in bone homeostasis. *Nat Med*. 2008; 14:299–305. [PubMed: 18297084]
120. Maier MM, Gessler M. Comparative analysis of the human and mouse Hey1 promoter: Hey genes are new Notch target genes. *Biochem Biophys Res Commun*. 2000; 275:652–660. [PubMed: 10964718]
121. Kohlhof H, Hampel F, Hoffmann R, Burtscher H, Weidle UH, Holzel M, Eick D, Zimmer-Strobl U, Strobl LJ. Notch1, Notch2, and Epstein–Barr virus-encoded nuclear antigen 2 signaling

- differentially affects proliferation and survival of Epstein-Barr virus-infected B cells. *Blood*. 2009; 113:5506–5515. [PubMed: 19339697]
122. Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, Kronenberg HM, Teitelbaum SL, Ross FP, Kopan R, Long F. Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. *Nat Med*. 2008; 14:306–314. [PubMed: 18297083]
123. Nevins AK, Thurmond DC. Caveolin-1 functions as a novel Cdc42 guanine nucleotide dissociation inhibitor in pancreatic beta-cells. *J Biol Chem*. 2006; 281:18961–18972. [PubMed: 16714282]
124. Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J Cell Biochem*. 1997; 66:1–8. [PubMed: 9215522]

Fig. 1.

Establishment of the ST2/Rx2^{dox} sub-line with conditional Runx2 expression. **a** ST2/Rx2^{dox} cells were treated with the indicated concentration of Dox for 48 h, and whole cell extracts were subjected to Western blot analysis with either anti-FLAG antibodies to detect FLAG-Runx2 or with anti-tubulin antibodies as control. **b** ST2/Rx2^{dox} cells were treated with 250 ng/mL Dox for 48 h and subjected to RT-qPCR analysis to of Runx2 mRNA. **c** ALP staining of ST2/Rx2^{dox} cells following Dox treatment for 48 h. **d** ST2/Rx2^{dox} cells were treated with Dox for 48 h and the mRNA level of the indicated genes assessed using RT-qPCR analysis. Bars represent the mean±SD ($n=3$) from a representative experiment, which was repeated at least three times with similar results. Transcript changes detected by RT-qPCR were significant with $p < 0.05$. GAPDH was used for normalization, and its expression did not significantly change by Dox treatment

Fig. 2. Unsupervised hierarchical clustering of Runx2-regulated genes. **a** Hierarchical clustering was carried out using the Minkowski distance function and the Ward clustering method for 216 genes that Runx2 stimulated or inhibited by 2.5 fold with $p < 0.008$ on either day 1 or day 2. The heat map represents z-scores across all 16 samples. **b** Heat map showing the z-scores for 20 upregulated and 20 downregulated genes with the most significant changes

Fig. 3. Runx2 decreases ST2 cell proliferation. **a** RT-qPCR analysis of IGF1 mRNA in ST2/Rx2^{dox} cells treated with Dox or vehicle. **b** MTT-based cell proliferation assay of ST2/Rx2^{dox} cultures treated with Dox or vehicle for the indicated time periods. **c** Representative phase contrast micrographs showing ST2/Rx2^{dox} cells after 24 and 48 h of treatment with vehicle or Dox

Fig. 4.

Runx2 promotes osteoclastogenic potential of ST2 cells. **a** Primary mouse splenocytes were co-cultured with either the parental ST2 or ST2/Rx2^{dox} cells in the presence of Dox or vehicle. Osteoclasts are demonstrated by TRAP staining of day 13 cultures after collagenase digestion and washing of the ST2 cells. **b** Quantitation of TRAP-positive area in the same co-cultures (mean $\pm$ SD, $n=4$). **c–e** RT-qPCR analysis of Runx2-regulated genes related to osteoclastogenesis. RT-qPCR data are presented as the mean $\pm$ SD. All p values were <0.05 , except for Hey1 where $p=0.16$.

Fig. 5.

Genes involved in Runx2-mediated osteoblast-driven osteoclastogenesis. The model shows Runx2-stimulated pro-osteoclastogenic genes on the *left* and Runx2-inhibited anti-osteoclastogenic genes on the *right*. A hypothetical Runx2-driven self-limiting negative feedback loop is described at the *top* based on the microarray data and the reported inhibitory effects of the Notch pathway on Runx2 and on osteoclast differentiation.

Table 1

Runx2-responsive genes related to osteoblast differentiation

Gene name	<i>p</i> value	Fold change	
		Day 1	Day 2
Bglap2, Bone gamma-carboxyglutamate protein 2	7.9E-11	12.7	17.8
Akp2, Alkaline phosphatase 2, liver	3.1E-07	4.1	5.8
Runx2, exon 9 (Chr 17)	3.2E-09	10.9	4.9
Sp7, (Osterix), Trans-acting transcription factor 7	2.1E-06	3.3	4.0
Col24a1, Collagen, type XXIV, alpha 1	9.8E-06	2.6	3.7
BSP (Ibsp), Integrin binding sialoprotein	2.3E-07	0.87	3.7
Mglap, Matrix gamma-carboxyglutamate protein	1.7E-13	-4.2	-8.8
Mustn1, Musculoskeletal, embryonic nuclear protein 1	7.9E-11	-2.3	-10.4

Fold change refers to average gene expression in Dox-treated versus control cultures ($n=4$). Negative values represent downregulation by Dox. Statistical significance (p values) was calculated for the changes on days 1 and 2 combined

Table 2

Runx2-responsive genes related to osteoblast proliferation

Gene name	<i>p</i> value	Fold change	
		Day 1	Day 2
Tspan32, Tetraspanin 32	1.8E-07	3.5	4.4
Megf10, Multiple EGF-like-domains 10	2.9E-09	3.3	4.0
Capn6, Calpain 6	1.8E-06	1.5	3.8
Mcm5, Minichromosome maintenance deficient 5	1.1E-08	2.0	2.6
E2f2, E2f transcription factor 2	6.2E-03	1.7	2.2
IGF2, Insulin-like growth factor 2	1.2E-06	-1.4	-2.8
Casp4, Caspase 4	4.3E-06	-2.3	-2.8
Igf2bp3, Igf2 mRNA binding protein 3	1.9E-05	-1.9	-3.0
Fg11, Fibrinogen-like protein 1	1.1E-05	-1.9	-3.1
Igf2bp2, Igf2 mRNA binding protein 2	2.4E-08	-2.2	-3.2
Igf1, Insulin-like growth factor 1	4.1E-10	-3.6	-5.4

Fold change refers to average gene expression in Dox-treated versus control cultures ($n=4$). Negative values represent downregulation by Dox. Statistical significance (p values) was calculated for the changes on days 1 and 2 combined

Table 3

Runx2-responsive genes related to osteoclastogenesis

Gene name	<i>p</i> value	Fold change	
		Day 1	Day 2
Hey1	4.1E-10	8.5	10.2
Ltc4s, Leukotriene C4 synthase	4.1E-10	8.1	7.2
Tnc, Tenascin c	1.4E-09	6.7	6.0
Apcdd1, Adenomatosis polyposis coli downregulated 1	5.0E-06	2.7	5.0
Sema7a, Semaphorin 7A	1.2E-07	4.6	4.7
Cdk5r1, Cyclin-dependent kinase 5, regulatory subunit (p35) 1	1.3E-07	2.8	4.4
Efnb1, EphrinB1	1.3E-06	2.5	2.9
Sema6b, Semaphorin6B	0.04271	-1.4	-1.5
Sema3f, Semaphorin3 F	1.2E-06	-2.1	-2.4
Sema3a, Semaphorin 3A	1.2E-07	-2.8	-2.4
OPG, Tnfrsf11b, Osteoprotegerine	4.1E-09	-3.4	-3.3
Il1rn, Interleukin 1 receptor antagonist	0.00015	-3.2	-3.9
Npr3, Natriureticpeptide receptor 3	1.2E-07	-3.7	-4.8
Ogn, Osteoglycin	4.1E-10	-4.1	-4.9
Clec2d, Ocil, C-type lectin domain family 2, member d	4.8E-07	-5.0	-6.1
Rspo2, R-spondin 2 homolog (<i>Xenopus laevis</i>)	4.2E-08	-4.9	-6.1
Slco2b1, Solute carrier organic anion transporter family, 2b1	4.5E-06	-3.8	-6.3

Fold change refers to average gene expression in Dox-treated versus control cultures ($n=4$). Negative values represent downregulation by Dox. Statistical significance (p values) was calculated for the changes on days 1 and 2 combined