Transcriptional program of mouse osteoclast differentiation
governed by the macrophage colony-stimulating factor
and the ligand for the receptor activator of NFκB

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Cytokines macrophage colony stimulating factor (M-CSF) and the receptor activator of NFκB ligand (RANKL) induce differentiation of bone marrow hematopoietic precursor cells into bone-resorbing osteoclasts without the requirement for stromal cells of mesenchymal origin. We used this recently described mouse cell system and oligonucleotide microarrays representing about 9,400 different genes to analyze gene expression in hematopoietic cells undergoing differentiation to osteoclasts. The ability of microarrays to detect the genes of interest was validated by showing expression and expected regulation of several osteoclast marker genes. In total 750 known transcripts were up regulated by ≥ 2-fold, and 91% of them at an early time in culture, suggesting that almost whole differentiation program is defined already in pre-osteoclasts. As expected, M-CSF alone induced the receptor for RANKL (RANK), but also, unexpectedly, other RANK/NFκB pathway components (TRAF2A, PI3-kinase, MEKK3, RIPK1), providing a molecular explanation for the synergy of M-CSF and RANKL. Furthermore, interleukins, interferons and their receptors (IL-1α, IL-18, IFN-β, IL-11Rα2, IL-6/11R gp130, IFNγR) were induced by M-CSF. Although interleukins are thought to regulate osteoclasts via modulation of M-CSF and RANKL expression in stromal cells, we showed that a mix of IL-1, IL-6, and IL-11 directly increased the activity of osteoclasts by 8.5-fold. RANKL induced about 70 novel target genes, including chemokines and growth factors (RANTES, PDGFα, IGF1), histamine and α1A-adrenergic receptors, and three waves of distinct receptors, transcription factors and signaling molecules. In conclusion, M-CSF induced genes necessary for a direct response to RANKL and interleukins, while RANKL directed a three-stage differentiation program and induced genes for interaction with osteoblasts, and immune and nerve cells. Thus, global gene expression suggests a more dynamic role of osteoclasts in bone physiology than previously anticipated.
Introduction

Bone is a dynamic tissue that is constantly remodeled, *i.e.* degraded and renewed. These two processes are accomplished by two main types of bone cells: bone-forming osteoblasts, of mesenchymal origin, and bone-resorbing osteoclasts, of hematopoietic origin (1). Understanding the generation and activation of these two cell types will help to unravel many processes involved in bone metabolism and remodeling. This knowledge may be used to develop novel, better treatments for osteoporosis, a disease prevalent in old age and characterized by bone loss and high risk of fractures. Osteoclast generation (osteoclastogenesis) is a multi-step process that can be reproduced *in vitro*. The *in vitro* osteoclastogenesis systems used to comprise mixtures of stromal or osteoblastic cells together with osteoclast precursors from bone marrow (2, 3). In such systems, stromal / osteoblastic cells are usually stimulated by 1α,25-dihydroxyvitamin D₃ to produce factors that support osteoclast formation. More recently, autonomous mouse osteoclastogenesis systems have been developed using bone marrow cells cultured with soluble forms of the cytokines M-CSF (macrophage-colony stimulating factor) and a soluble form of RANKL (receptor activator of nuclear factor κB ligand) (4, 5). These two cytokines are now recognized as the major factors contributed by stromal cells / osteoblasts for support of osteoclastogenesis (reviewed in 6). Therefore, their addition to the culture medium overcomes the need for stromal cells.

M-CSF, a homodimeric cytokine of the colony-stimulating factor family, is well known for its ability to stimulate proliferation and subsequent differentiation of cells of the macrophage / osteoclast lineage (7, reviewed in 8). The recently identified cytokine RANKL, also named ODF, OPGL or TRANCE (4, 9), is a member of the family of tumor necrosis factor (TNF)-like transmembrane or soluble cytokines, which usually act as trimers. RANKL has been identified and characterized as a long-sought factor stimulating osteoclast development (10). Expression of the receptor for M-CSF (c-Fms) is a crucial feature of an osteoclast precursor. The addition of M-CSF to osteoclast precursors induces the expression of the receptor for RANKL (RANK), which together with cell adherence allows differentiation to proceed (11, 12). The main features of intracellular signaling by c-Fms and RANK have been elucidated: c-Fms is a transmembrane tyrosine-specific protein kinase receptor, whose intracellular signaling involves Src and MAP kinase Erk activation (13; 14). RANK is a member
of the TNF receptor superfamily (15), and, like other family members, signals to the activation of the transcription factors NFκB and, through the kinase Jnk, c-Jun (16, reviewed in 6). Thus, the present knowledge on signaling by M-CSF and RANKL does not distinguish them from the other family members. The question remained open on how these two cytokines can drive such a complex and a specific process, such as the formation of strongly adherent, large, multinucleated bone-resorbing cells from a non-adherent, heterogeneous population of small bone marrow cells. Therefore, to better understand the process of osteoclastogenesis at the molecular level, we have analyzed gene expression patterns induced by M-CSF and RANKL during mouse osteoclast differentiation, using oligonucleotide microarrays representing about 9,400 mouse genes. The patterns of gene induction and their identity provide a basis for further molecular examination of osteoclast formation.

Experimental Procedures

In vitro mouse osteoclastogenesis

Cytokine-driven stromal cell-free mouse osteoclastogenesis was done as described by Shevde et al. (5). Briefly, bone marrow was collected from about twenty 5-weeks old male mice (C57BL, MA612, TIF/SPF). The cells were plated on 10-cm tissue culture dishes at 2x10^8 cells/dish, in 20 ml medium with 10% fetal calf serum (FCS) and incubated overnight. Next day, only non-adherent cells were collected, washed with PBS (without Ca^{2+} and Mg^{2+}) and purified by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech). For TRAP staining and pit assays, isolated non-adherent bone marrow cells were plated on 48-well plates at 1x10^5 cells/well in 1 ml, without or with dentine slices, respectively. For RNA extraction, about 8x10^6 isolated non-adherent bone marrow cells were plated in a 10 cm dish. The following cytokines (all from R&D Systems, UK) were added: 10 ng/ml recombinant mouse M-CSF, 30 ng/ml recombinant mouse His-RANKL (Cat. No. 462-TR) and 2.5 µg/ml anti-His antibodies (for RANKL cross-linking). The cultures were maintained typically for up to 11 days and re-fed twice weekly by medium semi-depletion. The absence of stromal cells in this culture system stems from the isolation of non-adherent cell population after 1 day incubation in medium with FCS, after which stromal cells are separated as an adherent population. Furthermore, no stromal cell layer is formed.
during culturing and the system was not stimulated with the active form of vitamin D₃, which acts via stromal cells (data not shown).

RNA was extracted from the total population of unstimulated non-adherent cells (day 0) or from the adherent cell population after 1, 3, or 6 days of stimulation with the cytokines (day 1, 3, 6). Both M-CSF alone and M-CSF plus RANKL induced adhesion of a subpopulation of non-adherent bone marrow cells already after 1 day of treatment. This allowed the selection of cells of monocyte-macrophage / osteoclast lineages. Practically no cells became adherent in medium containing 10% FCS without the cytokines.

**TRAP cytochemical staining and pit assay**

TRAP staining of adherent cultures was done with a kit from Sigma (Switzerland) exactly according to manufacturer's instruction (Procedure No. 386). The stained cells developed red color of different intensity. Pit assay with dentine slices was done as previously described (3) and pits were stained with toluidine blue. The cells were plated on the dentine slices from the beginning of the culture. Microphotography was done with the camera Nikon FM2 and film Kodak Gold Ultra 400. The microscope for cell culture photographs was Zeiss Axiovert 100 and for bone slices Leitz Laborlux S. The quantitative image analysis for pit area was done with the Leica Qwin Image Processing and Analysis Software.

**RNA isolation**

Cells have been harvested in a guanidinium isothiocyanate-containing buffer and total RNA has been extracted, treated with DNAse I and purified according to the manufacturer’s instructions (RNeasy mini kit, QIAGEN).

**Quantitative radioactive RT-PCR**

This PCR method was used to quantify the expression of marker genes prior to microarray analysis, as well as to confirm the regulation of some genes identified by microarray analysis. First strand complementary DNA was synthesized according to standard protocols. Briefly, for each sample, 10 units of RNase inhibitor (ROCHE Molecular Diagnostics) and 100 ng of random hexanucleotides (Amersham Pharmacia Biotech) were added to 1 µg of DNase I-treated total cellular RNA. The samples were subjected to a denaturation step of 5 min at 65°C, chilled on ice and filled to 20 µl with a nuclease-free solution containing another 10 units of RNase inhibitor, 2.5 mM of each dNTP, 50 mM Tris-HCl pH 8.3, 60 mM KCl, 10 mM MgCl₂ and 1 mM DTT. The mixture was supplemented (cDNA) or not (RT-) with 20
units of Avian Myeloblastosis Virus reverse transcriptase (Stratagene). Subsequently, the samples were incubated for 2 h at 42°C, then 5 min at 95°C, diluted 5-fold with nuclease-free water, and stored at -80°C until use. One µl of these diluted cDNA or RT(-) controls was subjected to PCR amplifications, carried out in a final volume of 25 µl. The PCR reaction mixture contained 100 µM of each dNTP, 1 µCi of α[32P]-dATP, 1 µM of each primer and 1.25 units of “hot start” thermostable DNA polymerase and corresponding reaction buffer (FastStart Taq, ROCHE Molecular Diagnostics). The amplification protocol consisted of an initial step of 5 min at 95°C, 12-34 cycles of denaturation at 94°C for 1 min, annealing at 57/60°C (all genes at 57°C, except c-fms, at 60°C) for 1 min, and extension at 72°C for 1 min 20 s. The amplification was terminated following a final incubation step at 72°C for 10 min. Aliquots of PCR products, supplemented with a loading buffer (final concentrations: 5% glycerol, 10 mM EDTA, 0.01% SDS, 0.025% xylene cyanol and bromophenol blue dyes), were fractionated on 8% polyacrylamide gels. The gels were vacuum-dried, exposed to phosphor-storage screens (Molecular Dynamics) and imaged by PhosphorImager (Molecular Dynamics). The signals on images were quantified by the ImageQuant software (Molecular Dynamics).

The primers (forward and reverse, given in the 5’ to 3’ orientation) and the number of cycles used in PCR are listed below. For each gene, a cycle curve experiment was performed and the optimal number of PCR cycles was chosen according to its position in the middle of the linear range of amplification.

| Gene                  | Primers                                | Cycles |
|-----------------------|----------------------------------------|--------|
| c-fms                 | AGCTCTCAGTACTTACGGGCAAAAGGCACGGGCTCCTAGA | 25     |
| Receptor activator of NF-κB (RANK) | TTTGTGGAATTGGGTCAATGAT and ACCTCGCTGACCAGTGTGAA | 26     |
| Tartrate-resistant acid phosphatase (TRAP) | GACGATGGCGCCTGACTTCA and GCGCTTGGAGATCTTAGTGTGA | 26     |
| Cathepsin K (CathK)    | ACGGAGGCATCGACTCTCGAA | 26     |
GATGCCAAGCTTGCGTCGAT, 28 cycles;
Calcitonin receptor (CalcR):
GACAACTGCTGGCTGAGTG and GAAGCAGTAGATAGTCGCCA, 34 cycles;
c-src:
CCAGGCTGAGGAGTGGTACT and CAGCTTGCGGATCTTGTAGT, 29 cycles;
Integrin β3 (ITGβ3):
ATTGAGTTCCCAGTCAGTGAG and GACAGGTCCATCAAGTAGTAG, 30 cycles;
TATA box binding protein (TBP):
AGTGAAGAACAATCCAGACTA and CCAGGAATAATTCTGCTCAT, 26 cycles;
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH):
CTGCACCACCAACTGCTTAG and AGATCCACGACGGACACATT, 19 cycles;
18S ribosomal subunit RNA (18S):
CCTGGATACCGCAGCTAGGA and GCGGCGCAATACGAATGCC, 12 cycles.
Histamine receptor H2 (H2R):
CTGCCATTTACCAGTTGTCC and CTTTGCACTTGAAGGTGTCAT, 34 cycles;
Circadian locomoter output cycles kaput (CLOCK):
ATCTGCTGGAAAGTGACTCAT and ATTTGGTTCTTCAACAGTACAC, 29 cycles;
LIM domain cysteine rich protein 1 (CSRP):
GCAGGCACGCTGAGCACA and CATCGGAAGCAGGACCTTATG, 31 cycles.

**Gene expression determination using high-density oligonucleotide microarrays**

The data described are derived from 6 independent primary culture preparations that contained various treatments or controls and were analyzed on 13 microarrays. Except for RNA from day 0 control, total RNA from each sample was extracted separately and analyzed by microarray individually. Due to low yields, the RNA from day 0 was a pool from 3 independent cultures. For treatment with M-CSF alone, samples were
from 2 (day 1) or 1 determination (days 3 and 6). Treatment with M-CSF and RANKL comprised 2 (day 1) or 3 (days 3 and 6) determinations. The data are represented in several analysis groups (A-G) to show experimental variability, time course of gene changes and different modes of data normalization (relative to day 0 or, for M-CSF plus RANKL, relative to time-matched treatment with M-CSF alone).

We have assessed gene expression patterns of approximately 9,400 genes during primary mouse osteoclasts differentiation (~5,700 functionally characterized genes and ~3,700 EST clusters) using microarrays consisting of coated glass slides on which series of oligonucleotide probes have been synthesized in situ (Affymetrix GeneChip® Murine Genome U74A arrays). Biotin-labeled cRNA probes were generated from each sample to be analyzed, starting from 5µg of DNAse I-treated total cellular RNA prepared as described above. The cRNA probes were individually hybridized on the arrays and the signals were detected according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA, USA).

**Analysis of gene expression on microarrays**

The hybridization data were analyzed using the software provided by Affymetrix (MAS4.0) and the software Expressionist 3.0 (GeneData, Basel, Switzerland). Hierarchical and nonhierarchical (SOM, self organizing maps) clustering of up regulated genes was performed using the latter software, in order to group the genes, or sort genes in a given functional group, according to patterns of expression. Genes were considered as expressed, if they were classified as P - present (and not M - marginal or A - absent) at least at one time point in a time course analysis group. The genes presented in detail were all detected with gene-specific probe sets and not with sets that could recognize gene families. Expression levels below 20 units were tresholded to 20, since discrimination below this value cannot be performed with confidence, and fold-regulation factors (fold induction or repression) were clamped to 10-fold. Genes were selected as regulated by a given treatment if their expression deviated more than 2-fold from the corresponding control. This threshold reflected 2 SEM (standard error of the mean) intervals around the mean relative levels of all 7545 (day 3) or 6895 (day 6) expressed genes in cells treated with M-CSF and RANKL (SEM=0.43 and 0.50, respectively for day 3 and day 6, n=3 for each). In addition to this statistical criterion, the biological data indicated that 2-fold was a meaningful difference, since osteoclast-specific genes (RANK, Mitf and Atp6i) were also induced to a similar degree (see the Results section and Fig. 1). As several determinations
were available, the criteria for gene selection were extended to include 2-fold regulation in both (day 1) or in 2 out of 3 experiments (days 3 and 6). When calculating average expression from several experiments, the median value was used preferentially, if the samples number allowed it (n=3). For duplicate samples, the mean value was calculated.

**Real-time PCR**
This technique was used to confirm the expression patterns of some genes identified by microarrays analysis. Briefly, 1 µl of cDNAs or RT(-), obtained as described in previous sections, served as a template in PCR reactions performed in a final volume of 50 µl. Specific primer pairs were designed for the genes of interest and for GAPDH, which was used as an internal control for normalization. The SyBr Green PCR Core Reagents system (Perkin-Elmer Applied Biosystems) was used for real-time monitoring of target sequence amplification by the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The amplification program comprised an initial step of 10 min at 95°C, and up to 40 cycles consisting of denaturation at 95°C for 30 s and annealing/extension at 60°C for 1 min. Negative controls were included in each PCR series, with RT(-) in place of a cDNA sample. The primers (forward and reverse, given in the 5’ to 3’ orientation) are listed below.

| Gene        | Primers:                                                                 |
|-------------|--------------------------------------------------------------------------|
| PDGF-α      | CCACATCGGCCAACCTTCC and ACAACAGCCAGTGCAGCG;                               |
| ScyA5/RANTES| TCCAATCTTGCAAGCTGTGTTTG and TTGAACCCACTTCTTCTCTGGGTT.                     |

**Results**

**Cellular and molecular characterization of primary mouse osteoclasts**
Isolated non-adherent mouse bone marrow mononuclear cells cultured *in vitro* in the presence of M-CSF and RANKL became adherent after 1 day and exhibited phenotypic features of osteoclasts after 3-11 days. The cells were adherent, positive for tartrate-resistant acid phosphatase (TRAP) staining, often multinucleated, and they
acquired a bone-resorbing activity in the pit assay (Fig. 1a). M-CSF alone induced formation of adherent, weakly TRAP-positive cells with no bone resorptive activity (Fig. 1a), while in the absence both factors, no adherent cells were detected after up to 8 days (data not shown). Further analysis included unstimulated non-adherent mononuclear cell population (day 0), and M-CSF alone or M-CSF plus RANKL-stimulated adherent cultures (days 1, 3 and 6).

[FIG. 1]
Within 3 to 6 days of culture with M-CSF and RANKL, messenger ribonucleic acids (mRNAs) for seven known osteoclast markers were up regulated in the adherent cell population: c-fms, RANK (encoding receptors for M-CSF and RANKL), integrin β3 (ITGB3), calcitonin receptor (CalcR), TRAP, cathepsin K (CathK), and c-src (Fig. 1b). Although, as expected, M-CSF alone could not induce a full osteoclast phenotype, it could time-dependently induce three markers of the osteoclast lineage (c-fms, RANK and ITGB3, Fig. 1b). There was also a weak induction of TRAP and c-src, mainly at day 6 (Fig. 1b). RANKL was added in the presence of M-CSF, which is necessary to prime the responsiveness to RANKL by inducing its receptor RANK (reviewed in 6, and Fig. 1b). RANKL induced another four osteoclast markers (CalcR, TRAP, CathK and c-src, Fig. 1b) above levels at day 1 and above time-matched M-CSF controls. The mRNA levels of housekeeping genes were similar in all conditions (Fig. 1b). We concluded that this mouse osteoclastogenesis system generates cells with cellular, functional and molecular features of osteoclasts. M-CSF and RANKL complement each other by inducing different sets of OC markers.

Gene expression profiling by DNA microarray analysis
To explore genome-wide gene expression patterns during mouse osteoclastogenesis, we extracted RNA at days 0, 1, 3 and 6 of the in vitro culture and analyzed them using oligonucleotide microarrays representing about 9,400 distinct mouse genes (Affymetrix GeneChip microarrays). The amounts of RNA required for microarray analysis and the yields that can be obtained from primary mouse osteoclastogenesis cultures are such that not all treatments can be done in each experiment. Therefore, we performed 6 primary cultures containing different combinations of treatments and analyzed them on 13 microarrays. This permitted the analysis of technical reliability, and of biological variability for the most important treatments, as treatments with M-CSF plus RANKL at days 3 and 6 were analyzed 3 times each from independent samples. Controls and day 1 treated groups were respectively from 1 and 2 replicates
To assess the sensitivity and accuracy of DNA microarrays for quantitative gene expression analysis, we first analyzed the microarray hybridization data obtained for the osteoclast markers and compared them to those obtained by RT-PCR (Fig. 1c). The microarray analysis detected three out of seven osteoclast markers (c-fms, RANK and TRAP), which, consistent with the RT-PCR, were found to be up regulated. Judging from threshold cycle numbers for detection by RT-PCR, these three markers were expressed more strongly than the others. The housekeeping genes were detected and found not to be up regulated, in agreement with the RT-PCR data. These results indicated that microarrays can detect the expression and regulation of osteoclast marker genes, albeit at a lower sensitivity than RT-PCR.

The expression of other genes characteristic for the osteoclast phenotype was further examined on microarrays. The Mitf gene encodes the microphthalmia-associated transcription factor, expressed in osteoclast progenitors and known to play a critical role in the maturation of osteoclasts, in cooperation with transcription factors PU.1 and c-Fos (17, 18), as illustrated by the consequences of mutations in human and mouse (19, 20). The Atp6i gene encodes a proton pump that is necessary for the resorption activity of osteoclasts, as demonstrated by its inactivation in mice and in a subset of human autosomal recessive osteopetrosis cases (21-23). The levels of Mitf and Atp6i transcripts were progressively up regulated (2-6-fold) by treatment of bone marrow cells with M-CSF and RANKL for up to 6 days (data not shown). Thus, the expression of these two genes further verified the osteoclast phenotype of generated cells and showed the feasibility of detection of osteoclast-related genes by microarrays.

[FIG. 2]

Up regulated expression of 750 known genes is associated with osteoclastogenesis

In the rest of the present study, we analyzed the expression of known genes and not of uncharacterized “expressed sequence tags” (ESTs). In particular, we focused on up regulated genes, expecting that the contributors to the osteoclast differentiation process would be among them. As a threshold for expression regulation, we selected a 2-fold difference from the control, a criterion with statistical and biological meaning (details in Experimental Procedures).
We detected 750 known genes that were induced at least 2-fold by M-CSF and RANKL at days 1, 3, or 6, relative to day 0. This subset of genes was further analyzed by a nonhierarchical clustering method, defining 10 classes of genes, grouped according to their temporal patterns of regulation (Fig. 2). There was a good concordance between the general gene expression patterns observed in independent primary cultures (Fig. 2, compare A, B and C). Already one day after stimulation, 39% of the genes were induced (clusters I-IV, representing a total of 296 genes), either transiently (clusters I-III) or continuously (cluster IV). The remaining genes were induced either at day 3 (clusters V-IX, 391 genes, 52 %), or at day 6 (cluster X, 63 genes, 9%). These data indicate that the major transcriptional changes take place before day 3 (91% of genes), which is the time when the osteoclast phenotype becomes detectable in the cultures (TRAP staining, bone-resorbing activity). Further progression of osteoclast differentiation after day 3 involved up regulation of few new genes and already expressed genes were either preserved or down regulated (Fig. 2). Thus, it appears that early commitment to the phenotype (attachment, cell morphology changes, up to day 3 in culture) already includes the expression of an almost complete new gene repertoire.

Analysis of the identity of the up- and down regulated genes led us to define several large groups according to their cellular function. Two major groups of down regulated genes were: a) positive regulators of cell cycle and b) lymphoid and erythroid markers (data not shown). These changes are consistent with the commitment of the analyzed cell population to differentiation along myeloid / osteoclastic lineage and with the loss of cells from lymphoid and erythroid lineages in the non-adherent cell fraction after cytokine stimulation. Two groups of up regulated genes with a function in cell adhesion / motility and in intracellular trafficking are not further discussed here, as they are expected, based on features of the osteoclast phenotype and probably do not have a major role in directing the differentiation process. Other major groups of identified up regulated genes, which are expected to contribute to differentiation process, are discussed below.

[FIG. 3]

M-CSF induces the up regulation of components of the signaling pathways of RANK and NFκB, interleukins, interferons and chemokines

We focused on genes up regulated with time in adherent cultures containing M-CSF, a survival and differentiation factor for the myeloid and osteoclast lineages. The
induction of these genes was calculated relative to day 0, which comprised undifferentiated non-adherent bone marrow mononuclear cells (Fig. 3a, b, left panels, Ref: 0). Therefore, an increase in gene expression may stem from an enrichment of certain mRNAs in the adherent cell population, relative to non-adherent bone marrow cells. These genes would reflect an expression pattern of an M-CSF-primed myeloid / osteoclast precursor. In addition, an increase in gene expression may stem from the M-CSF induction of specific genes or of stabilization of their mRNAs in a given myeloid / osteoclast precursor population. These two mechanisms cannot be distinguished, since adhesion is a part of M-CSF induced cell priming towards myeloid / osteoclast lineages.

The induction of genes shown in Fig. 3 was mainly dependent on M-CSF, less on RANKL. This is shown by the comparison of M-CSF-treated cultures to day 0 (Fig. 3a, b, right panels, Ref: 0) and by comparison of M-CSF plus RANKL-treated cultures to time-matched M-CSF-treated cultures (Fig. 3a, b, right panels, Ref: M). Among M-CSF-induced genes, two particularly interesting groups became apparent: one comprising molecules related to the signaling to NFκB (either form RANK or other receptors, Fig. 3a), the other comprising molecules related to signaling by interleukins, interferons, and chemokines (Fig. 3b).

The importance of NFκB for osteoclast formation was previously best documented by the osteopetrotic phenotype (high bone mass due to dysfunctional osteoclasts) observed in knockout mice (24, 25). RANK is the receptor for RANKL and its role in osteoclast differentiation and activation is well established in vitro, as well as in mice and in humans (15, 26-28). We have detected the induction of RANK, as previously reported (Fig. 1b,c; 11), but also of 8 other components of this or related signaling pathways. Some of them are expected to act as positive regulators (TRAF2A, PI3-kinase regulatory sub-unit PI3KR2, 1κB protein kinase IKK-i) (29-31), while the others are negative regulators of the RANK and NFκB pathways (TRAF-interacting protein TRIP, and the small G protein κB-Ras1) (32, 33).

Several cytokines, chemokines, their cognate receptors and associated signal transduction proteins were also induced in a time-dependent manner by M-CSF (Fig. 3b). Their induction was strong and persistent, reaching a maximum at 3 to 6 days (Fig. 3b, left panel). The function of most of these genes has already been linked to osteoclastogenesis. Some are expected to be positive regulators (IL-1α, IL-11 receptor α2 and co-receptor for IL-6/IL-11 gp130) (reviewed in 34), while others are
negative regulators of osteoclast differentiation (IL-18, IL-10 receptor α, interferon-β1, interferon-γ receptor or its signaling component STAT1) (35-37). In agreement with the observations made for RANK and NFκB signaling pathways, these results suggest a tight and balanced (both positive and negative) regulation of osteoclastogenesis by the induced genes.

Although IL-1, IL-6 and IL-11 are implicated in stimulation of osteoclastogenesis, their primary target cell is thought to be the stromal cell, which in turn regulates osteoclastogenesis by producing M-CSF and RANKL (10, 34). So far, the action of M-CSF and RANKL was studied only in the co-culture osteoclastogenesis systems. Here, we for the first time detected an increase in the expression of IL-1α, and the receptor components for IL-6 and IL-11 in differentiating osteoclasts in the absence of stromal cells. The significance of this increase was examined by measuring the effects of these interleukins in the stromal cell-free osteoclastogenesis system. The results in Fig. 3c (left panel) show that IL-1α had a mild stimulating effect on osteoclast formation (about 2-fold), while IL-6 and IL-11 were less active and did not synergize with IL-1α. However, these interleukins had a strong synergistic stimulatory effect on the bone resorption activity of osteoclasts (about 8.5-fold, Fig. 3c, right panel). IL-1α alone stimulated osteoclast activity about 4-fold, while IL-6 and IL-11 alone were without effect. These results show the biological significance of the increase in mRNA of IL-1α and the receptor components for IL-6 and IL-11 during stromal cell-free osteoclastogenesis. This unexpected stimulation of bone resorption activity unravels the osteoclast’s potential to directly respond to pro-resorptive cytokines. Furthermore, M-CSF and RANKL are not able to maximally stimulate differentiation and activity of osteoclasts, as generally assumed.

[FIG. 4]

**RANKL induces the up regulation of genes for growth factors, cytokines, chemokines, receptors, signaling molecules and transcription factors**

RANKL-induced genes were identified by normalizing gene expression profiles at specific time points in cultures treated with M-CSF and RANKL together to those in time-matched cultures treated with M-CSF alone (Fig. 4). Thus, in contrast to M-CSF-induced genes, RANKL-induced genes stem mainly from mRNA induction / stabilization in a given population of myeloid / osteoclast precursors. We identified 255 such RANKL-induced genes, among which 11 encoding cytokines, chemokines
or growth factors, 13 receptors, 27 signaling molecules and 27 transcription factors and modulators. Genes in these four functional groups were further analyzed by a clustering algorithm and sorted according to the chronology of induction (Fig. 4a-d). One subset of the up regulated cytokines, chemokines and growth factors is expected to have a role in osteoclast differentiation and motility (C-type lectin SCGF, allograft inflammatory factor 1 AIF1, and chemokines SCYA2, 5, 7 and 8) (38-40). The second subset of genes may have a role in regulating osteoblasts, known to express receptors for and respond to these factors (insulin-like growth factor 1 IGF1, platelet-derived growth factor alpha PDGF-α and keratoepithelin TGFBI/BIGH3) (41-43). The third subset of cytokines comprises tumor necrosis factor family member (TNFSF9) that may activate lymphocytes (44) (Fig. 4a).

Among RANKL-induced receptors, there are several G-protein coupled receptors (FPR1, EMR1, OPRS1, PTGER2, H2R and ADRA1A) (Fig. 4b). With the exception of the prostaglandin receptor EP2 (PTGER2), which is involved in bone resorption (45), for most of these receptors a link to osteoclast biology is not obvious at present. The most intriguing observation is the increased expression of two receptors for biogenic amines and neurotransmitters (H2R and ADRA1A, the receptors for histamine and epinephrine, respectively).

Among the RANKL-induced subset of signaling molecules (Fig. 4c), there are many protein kinases (LIMK1, DM15, NEK2, STK6, PLK, VRK1, PIM1, FGR/SRC2 and HIPK2), several docking proteins (GRB10, DOK2, DAB2), and small G proteins and their regulators (RRAS, ECT2, RACGAP1 and RALGDS). The expression of phospholipase D (PLD1), responsible for phosphatidylcholine hydrolysis and acting as a critical mediator in many cellular pathways, was reproducibly increased as well. Interestingly, in bone cells, PLD was so far implicated in induction of pro-resorptive cytokine IL-6 by prostaglandin or thrombin in osteoblasts (46), but there are no reports on PLD expression and role in osteoclasts. Finally, the suppressors of cytokine signaling, SOCS1 and 3, were also up regulated, indicating a balanced control of the osteoclastogenesis process.

Within the group of transcription factors and their modulators (Fig. 4d), some genes can be connected to bone biology (ETL1, RELB and NFκB2). ETL1, a member of the SWI2/SNF2 family of helicases and nucleic-acid-dependent ATPases, has been shown, through gene inactivation in the mouse, to be important for normal bone growth (47). RELB encodes a transcription activator that participates to the NFκB
transactivator complex \((48)\). Since NFκB, a target of RANK signaling \((49)\), is critical for osteoclast differentiation \((24, 25)\), \(RELB\) may thus have a role on osteoclastogenesis as well. Another gene of the NFκB family, \(NFκB2\), is also upregulated by RANKL treatment and is equally known to play a crucial role in osteoclast differentiation, together with \(NFκB\) \((24, 25)\). According to a recent communication, \(c-myc\) could also be involved in that process \((50)\). Finally, several other transcription factors have a possible role in cell differentiation or cell fate determination (glial cells missing homologue \(GCMb\), LIM domain gene \(CSRP\) and myocyte enhancer factor \(MEF2B\)) \((51-53)\).

**[FIG. 5]**

A simplified schematic overview of changes in gene expression observed during osteoclast differentiation, together with characteristic examples, is provided on Fig. 5. Of note is the stable gene up regulation induced by M-CSF and three waves of gene regulation induced by RANKL.

**Confirmation of the gene expression profiles by quantitative PCR**

As an independent confirmation of the gene expression patterns determined by microarray hybridization, we measured the expression levels of selected genes using SyBr Green-based fluorogenic real-time PCR or radioactive quantitative PCR. As exemplified for \(PDGF-α, Scya5/RANTES, H2R, CLOCK\) and \(CSRP\), the expression profiles obtained by microarray analysis and quantitative PCR were very similar (Fig. 6).

**[FIG. 6]**

**Discussion**

Our study provides a first comprehensive view of coordinated regulation of the transcriptional program induced by M-CSF and RANKL during \textit{in vitro} differentiation of mouse bone marrow precursor cells into bone-resorbing osteoclasts. Special features of this study are: a) use of primary cells and not cell lines; b) analysis of several independent cultures for most important samples; c) monitoring of differentiation process cytochemically, functionally and molecularly in each experiment; d) validation of the microarray data by comparison of marker genes by RT-PCR and microarrays; and e) setting the gene selection criteria based both on statistical and biological considerations. These features should ensure the technical and biological reliability of analyzed gene expression changes.
The critical involvement of M-CSF and RANKL and their cognate receptors, c-Fms and RANK, in osteoclast formation, is now well established (recently reviewed in 6, 10). However, little is known so far about the resulting molecular events that underlay the phenotypic changes from precursor cells into committed pre-osteoclasts and active functional osteoclasts. For the basic understanding of this differentiation system as well as for the identification of possible new drug targets for the prevention or treatment of bone loss, it is of high interest to characterize further the signaling networks that are involved in osteoclast formation or activation. We demonstrate herein that a functional genomics approach has the potential to uncover such biochemical circuits based on the examination of the cascade of regulated genes. One typical example is our identification of regulated expression of several RANK and NFκB signaling pathway components. Altogether, from about 100 genes whose regulation is explicitly shown in this study, besides osteoclast markers only about 6 other genes (RANK, IFNγ, c-myc, IL-1, IL-18 and IL-11 receptor) were reported as expressed or up regulated in osteoclasts, while about 90 other genes are reported here for the first time, annotated and sorted according to their expected function. This provides a valuable resource for future studies of the osteoclast.

We particularly focused our analysis on genes that are up regulated at one or more stages of osteoclast differentiation and could, therefore, have a positive role in that process. In this way, among about 9,400 genes analyzed by microarrays, we have identified 750 known genes. A vast majority of these genes is induced at early times during differentiation. Therefore, main transcriptional changes occur during commitment to osteoclast lineage and not during later stages of differentiation. Pre-osteoclasts, thus, express almost all genes necessary to perform a differentiation program.

Our analysis shows that the contribution of M-CSF alone is not only to induce the clonal expansion of bone marrow-derived osteoclast precursor cells, but also to prepare the cells to respond to the osteoclast-specific differentiating stimuli. This was illustrated by the M-CSF-induced expression of RANK and its associated signaling pathways components in precursor cells, enabling them to respond to RANKL. This gene expression profile is in agreement with the proposed role of M-CSF in osteoclastogenesis (7) and with the reported induction of RANK by M-CSF (11). While up regulation of RANK was previously reported (11), the up regulation of RANK / NFκB signaling components is a novel finding, which provides a molecular
explanation for synergy between M-CSF and RANKL. Most of these signaling molecules are induced early after addition of M-CSF, suggesting that they may be direct novel target genes of this cytokine. TRAF2 is an adapter protein that is recruited to activated RANK receptor and transduces the signal to activation of c-Jun N-terminal kinase (Jnk) and NFκB. Protein kinase MEKK3 is also known for activating the same pathway and phospholipid kinase for regulating it. Finally, a component of NFκB transcription factor activity (NFκB2) is also up regulated. Interestingly, some of these signaling components were reported to be up regulated by a pro-resorptive cytokine TNF-α (54). Thus, it appears that synergy between different osteoclast-stimulating cytokines and RANKL may be achieved by similar mechanisms.

In addition to the RANK signaling pathway, of key importance for osteoclastogenesis, M-CSF induced a number of pro-resorptive (some interleukins or their receptors / co-receptors) and anti-resorptive cytokines (some interleukins, interferons or their receptors and associated signaling molecules). Therefore, a fine balancing of the osteoclast differentiation process is suggested by the transcriptional program of bone marrow precursor cells treated with only a single cytokine, such as M-CSF. This notion is in agreement with conclusions of Atkins et al. (55), who studied expression of selected genes during human osteoclast formation in a co-culture with the ST-2 stromal cell line. However, a novel aspect of our study is the expression and the activity of these cytokines in stromal-free osteoclastogenesis system. We showed that IL-1α and the receptor components for IL-6 and IL-11 are up regulated by M-CSF in osteoclast precursors without additional mediation by stromal cells. Furthermore, IL-1, IL-6 and IL-11 together increased bone resorption activity of differentiating osteoclasts, again without mediation of stromal cells. This notion is in contrast with the currently prevailing “convergence hypothesis” (56), according to which all osteoclast regulatory factors (including IL-1, IL-6 and IL-11) converge in their regulation mechanism on the expression of RANKL, its decoy receptors ODF and M-CSF (10, 34) by the stromal / osteoblastic cells. Our findings highlighted an underestimated ability of osteoclasts and their precursors to directly respond to the regulatory cytokines and to produce them. Another example of a possible osteoclast’s independent action is the concomitant up regulation of the chemokine RANTES and its receptor CCR5, whose expression provides a basis for an autonomous regulation of osteoclast motility. Together, from these data a picture of osteoclast is emerging, in
which this cell type is more independent, versatile and dynamic than currently appreciated.

With the aim of getting more insights into the mechanisms of action of RANKL, we searched for genes whose expression was more specifically regulated by this cytokine. Currently, there are only two known RANK target genes: c-Jun (5) and FosL1 (57). In this study we identified about 70 novel RANK-responsive genes. We observed that, among the genes induced by RANKL, a significant proportion functions in controlling transcription. Among these transcription factors, several have or may have a role in cell differentiation and would, therefore, deserve further investigation to determine their contribution to osteoclast differentiation. Another prominent group of RANKL-induced genes encodes signaling molecules, whose connection to osteoclast differentiation or activity is less obvious. However, transcriptional modulators and signaling molecules share a remarkable feature, their coordinated patterns of regulation over the time. Indeed, in both groups, most of the genes are not up regulated by RANKL at all time points studied (day 1, 3 and 6), but rather in waves, peaking at day 1, 3, or 6 (Fig. 5). This observation does not only suggest that these genes may be involved in the control of different stages of osteoclastogenesis, but also allows the definition of at least three previously unidentified steps in the osteoclast differentiation process, based on a transcriptional fingerprint.

A series of chemokines, cytokines and growth factors genes are also induced by RANKL. Some of them may play a role in osteoclast differentiation or activation (e.g. C-type lectin SCGF and allograft inflammatory factor 1 AIF1) (38, 39), while some others may be involved in communication with other cell types. For example, one of the RANKL-induced cytokines, the tumor necrosis factor family member TNFSF9, acts on T lymphocytes (44), the cell type implicated in the regulation of osteoclast differentiation. Chemokines (SCYA2, 5, 7 and 8) act as chemoattractants (40), suggesting that osteoclasts could either regulate their own motility or recruit other cell types in the areas of bone resorption. Up regulation of PDGF-α and IGF-1, both known to stimulate proliferation and differentiation of osteoblast precursors (41, 42), implies a novel direct coupling between osteoclasts activation and osteoblasts recruitment. These factors were already known to be present in bone, but so far osteoclasts were not implicated as the cells producing them.
A significant number of genes encoding receptors are also induced by RANKL. One of them, encoding the prostaglandin receptor EP2 (PTGER2), has a proven role in osteoclastogenesis (45). Again, as for pro-resorptive cytokines, prostaglandin E2 is thought to act mainly via stromal cells / osteoblasts, but not directly on osteoclasts. Our identification of the up regulated prostaglandin receptor EP2 during osteoclast differentiation supports the unexpected notion that osteoclast can directly respond to prostaglandin E2.

Surprisingly, two receptors for biogenic monoamines were up regulated: the receptors for histamine (H2R) and the α1-adrenergic catecholamine receptor (ADRA1A). Biogenic monoamines are neurotransmitters and vasoactive substances released by the neurons in the central and peripheral nervous system. Bone is a well-innervated tissue where the nerve fibers come in a direct contact with osteoclasts (58). In addition, human and mouse osteoclasts have been recently reported to express mRNAs for axon guidance molecules, such as semaphorin 3B, suggesting that they can guide growing nerve fibers (59). We have also detected the expression of semaphorin 3B (data not shown). The up regulation of biogenic monoamine receptors during osteoclastogenesis indicates that osteoclasts, in addition to modulating neurite outgrowth, also have the potential to respond to stimuli released by the neurons. Histamine is also produced by the tissue mast cells, and the expression of its receptor in osteoclasts suggests that osteoclasts have a potential to respond to stimuli from the immune system as well. This regulation may have a pathophysiological and pharmacological relevance, since mast cell numbers strongly increase after ovariectomy, an in vivo situation of increased osteoclastogenesis and bone loss (60), and because histamine receptors antagonists can modulate osteoclast resorption in vivo (61).

In conclusion, by genome-wide identification of genes regulated during osteoclastogenesis, our study provides a basis for further molecular studies aiming at a better understanding of the osteoclast differentiation process. The identity of some of the genes described in this report sheds light on unsuspected potential of osteoclasts to regulate their own activity without mediation of stromal cells and to communicate with some other osseous and non-osseous cell types present in bone. Together, our data suggest that the cells of the osteoclast lineage have a role in bone physiology that is more dynamic and versatile than it is currently viewed.
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Footnotes

Abbreviations: DTT - dithiothreitol; EDTA – ethylenediamine-tetraacetic acid; EST – expressed sequence tag; FCS – fetal calf serum; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; IFN - interferon; IL - interleukin; M-CSF – macrophage-colony stimulating factor; PLD – phospholipase D; RANK - receptor activator of NFκB; RANKL - receptor activator of NFκB ligand; RT-PCR – reverse transcription – polymerase chain reaction; SDS – sodium dodecyl sulfate; TBP – TATA box binding protein; TNF – tumor necrosis factor; TRAP – tartrate-resistant acid phosphatase.

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Figure 1: Cytochemical, functional and molecular characterization of the mouse osteoclastogenesis system.

Mouse bone marrow mononuclear cells were cultured in the presence of M-CSF or M-CSF and RANKL for 3-11 days, as described in Experimental Procedures. The cultures were analyzed for osteoclast phenotype (a, TRAP staining and pit assay), expression of marker genes by RT-PCR (b) or by microarray analysis (c).

(a) Tartrate-resistant acid phosphatase staining (TRAP) and dentine resorption in a pit formation assay (PIT) were monitored at the indicated days of culture in the presence of M-CSF only (M) or M-CSF and RANKL (M+R). Arrows indicate multinucleated TRAP-positive cells and resorption lacunae (pits). (b) The cultures were treated with M-CSF only or M-CSF and RANKL for 1, 3, or 6 days, and total RNA was extracted. The expression of the indicated osteoclasts markers, housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; TATA box binding protein, TBP), and 18S ribosomal RNA (18S), was analyzed by quantitative radioactive reverse-transcription/polymerase chain reaction (RT-PCR). The radioactive PCR products were analyzed by polyacrylamide gel electrophoresis and imaging by PhosphorImager. Gel images obtained by PhosphorImager are shown. (c) RNA samples, including those described in (b), were analyzed by GeneChip microarray and the data were compared to quantitative radioactive RT-PCR. For both types of analyses, mRNA levels are expressed as mean fold-regulation from 3 separate cultures treated with M-CSF and RANKL, relative to the pool of unstimulated cells at day 0. The threshold detection in RT-PCR is indicated by the number of PCR cycles. For the GeneChip microarray analysis, detection is indicated by symbols on a scale ranging from "-" (no significant signal) to "+++" (signal > 1000).

Figure 2: Clustering of transcripts up regulated during mouse osteoclastogenesis.

Mouse bone marrow mononuclear cells were cultured in the presence of M-CSF and RANKL for 0, 1, 3, or 6 days, total RNA was extracted and analyzed by GeneChip microarray. The microarray data were analyzed by the Expressionist software and expressed as fold-regulation relative to the day 0 control (untreated cells). Fold-regulations are presented in a black-red-green color code, as indicated at the bottom. The data from 9 chips are organized into 3 analysis groups (A-C) to show changes in gene up regulation with time and variation between experiments. Day 0 (a pool from
3 independent cultures) was the same for all groups, day 1 was different in A and B, while their mean is displayed in C, and days 3 and 6 were different for A, B and C. The 750 up regulated transcripts were nonhierarchically clustered, based on the temporal similarity of expression profiles. Expression profiles are shown in the three groups of columns on the left and ten gene clusters are shown on the right, sorted according to the peak time of induction (from early to late). The gene cluster graphs are derived from the average expression profile for all the genes in the corresponding cluster. The numbers in brackets next to each cluster represent the number of genes in the given cluster.

**Figure 3:** Expression profiles for genes preferentially induced by M-CSF.

Mouse bone marrow mononuclear cells were cultured in the presence of M-CSF only or M-CSF and RANKL for 0, 1, 3, or 6 days, total RNA was extracted, and analyzed by GeneChip microarray and the Expressionist software. The data are expressed as fold-regulation relative to the corresponding control, in a black-red-green color code, as indicated at the bottom. **Left panels:** Median fold-regulation of genes expression by M-CSF and RANKL, relative to the day 0 control (Ref: 0). The data are derived from three analysis groups (A-C, as in Fig. 2). **Right panels:** Fold-regulation of genes expression by M-CSF alone or M-CSF and RANKL, expressed either relative to the day 0 control (Ref: 0) or relative to M-CSF as a time-matched control (Ref: M). The latter comparison shows the contribution of RANKL. The data are derived from one analysis group, containing 1 determination per treatment (analysis group D). (a) Genes from RANK/NFκB signaling-related group. (b) Genes from interleukins (IL), interferons (IFN), and chemokine signaling-related group. (c) Mouse bone marrow mononuclear cells were cultured in 48-well plates in the presence of 10 ng/ml M-CSF and 30 ng/ml RANKL and with the addition of indicated interleukins at 1 ng/ml. TRAP staining was done after 6 days (n=4) and pit assay after 12 days (n=2). The results were quantified using a Leica image analysis system and are presented as percent of control (means ± SEM). **Left panel:** number of multinuclear TRAP-positive cells (MNC No.). **Right panel:** bone resorption (pit) area. The control number of multinuclear TRAP-positive cells was 259 ± 21 and the control pit area was 2.1 ± 0.5% (mean ± SEM).

**Figure 4:** Expression profiles for genes preferentially induced by RANKL.
Mouse bone marrow mononuclear cells were cultured in the presence of M-CSF only or M-CSF and RANKL for 1, 3, or 6 days, total RNA was extracted and analyzed by GeneChip microarray and the Expressionist software. The data are expressed as fold-regulation by M-CSF plus RANKL relative to M-CSF alone as a time-matched control, yielding the separate contribution of RANKL in the presence of M-CSF. The black-red-green color code for fold-regulation is indicated at the bottom. The data shown correspond to three analysis groups (A-C, Fig. 2), and are here expressed relative to M-CSF alone (analysis groups E-G). (a) Genes from cytokines, chemokines and growth factors group; (b) Genes from receptors group; (c) Genes from signaling molecules group; (d) Genes from transcription factors and modulators group.

**Figure 5:** Schematic representation of the time courses of gene expression in response to M-CSF and RANKL.

The data from Figures 3 and 4 are shown here as simplified time course graphs, representing qualitative changes in gene expression (elevated line for ≥2-fold increased expression). Examples of some regulated genes are shown under the line for each gene group. This representation allows seeing synchronized, stable up regulation of gene expression by M-CSF and three waves of gene induction initiated by RANKL. C: the corresponding control, as indicated in Figures 3 and 4.

**Figure 6:** Verification of microarray-based gene expression profiles by real-time PCR.

Mouse bone marrow mononuclear cells were cultured in the presence of M-CSF only or M-CSF and RANKL for 1, 3, or 6 days, total RNA was extracted and analyzed by real-time fluorogenic (°) or radioactive (*) quantitative PCR (qPCR). The RNA samples were identical to those analyzed by microarrays, as shown on the right panels in Figure 3. Data from both the qPCR assay and microarray hybridizations were normalized with GAPDH and plotted relative to the level in M-CSF treated cells at day 1. The effects of M-CSF alone or in combination with RANKL are illustrated at each time point (day 1, 3 and 6). Days and treatments are indicated at the bottom of each series of graphs.
Fig. 1

(a) M M+R M M+R
Day
3-4
5-7
8-11
TRAP
PIT

(b) Days
1 3 6
M-CSF RANKL

M-CSF-induced
RANKL-induced
Housekeeping

RT (-)

| Gene       | Radioactive RT-PCR | GeneCHIP |
|------------|--------------------|----------|
|            | Detection (cycle n°) | Regul. by M+R* | Detection | Regul. by M+R* |
|            | Day 3 | Day 6 | Day 3 | Day 6 |
| c-fms      | 20    | 6.3   | 4.5   | +++  | 2.9  | 3.3   |
| RANK       | 22    | 10.4  | 10.1  | +    | 2.4  | 3.2   |
| ITGB3      | 25    | 2.2   | 1.5   | -    | N.C. |
| CalcR      | 30    | 40.4  | 6.9   | -    | N.C. |
| TRAP       | 22    | 11.2  | 3.5   | ++   | 9.3  | 3.9   |
| CathK      | 26    | 16.5  | 5.7   | -    | N.C. |
| c-src      | 27    | 4.2   | 5.2   | -    | N.C. |
| GAPDH      | 17    | 1.4   | 1.3   | +++  | 0.8  | 0.8   |
| TBP        | 23    | 0.6   | 0.5   | +    | 0.7  | 0.5   |

* Mean fold regulation from 3 experiments, relative to day 0
- , no signal; +, signal<200; ++, 200<signal<1000 ; +++ , signal>1000
N.C., not calculated
Fig. 2

Cluster I (45)
Cluster II (61)
Cluster III (65)
Cluster IV (152)
Cluster V (22)
Cluster VI (46)
Cluster VII (139)
Cluster VIII (58)
Cluster IX (99)
Cluster X (63)

Fold changes

Days 0 1 3 6

A                 B                 C

750 up-regulated transcripts
**Fig. 3**

### RANK and NFκB-related

| Group | A-C | D |
|-------|-----|---|
| Days  | 0   | 1 | 3 | 6 | 0 | 1 | 3 | 6 |
| M-CSF | +   | + | + | - | + | + | + | + |
| RANKL | -   | + | + | - | + | + | - | + |
| Ref:  | 0   | 0 | 0 | 0 | 0 | M | 0 | M |

**Function**

- **RANK**: TNF receptor superfamily (TNFRSF), member 11a
- **TRAF2**: TNF receptor-associated factor 2. Activates JNK, NFκB
- **TRIP**: TRAF-interacting protein. Inhibits NFκB, via TRAF2
- **PI3KR2**: PI3K, regulatory subunit 2. Recruits the catalytic subunit
- **IKK-i**: Inducible IκB kinase. Activates NFκB
- **MEKK3**: Protein kinase, MAPK cascade. Activates NFκB and JNK
- **RIPK1**: TRAF-interacting protein. Inhibits NFκB, via TRAF2
- **KB-Ras1**: NFκB2. Transcription factor, DNA-binding subunit

### Interleukins, interferons, chemokines

- **IL1**: IL-1α. Stimulates OC-genesis
- **IL1RA2**: IL-11 receptor α2. Stimulates OC-genesis
- **GP130**: Component of IL-6/11 receptors signaling
- **IL18**: IL-18. IFN-γ inducing factor. Inhibits OC-genesis
- **IGIFBP**: IFN-γ inducing factor binding protein. Binds to IL-18
- **IL10R**: IFN-γ receptor. Inhibits OC-genesis
- **IFNB1**: IFN-β1. Inhibits T lymphocytes triggered OC-genesis
- **IFNGR**: IFN-γ receptor. Inhibits OC-genesis
- **IRF7**: IFN regulatory factor 7. Transcriptional activator
- **STAT1**: Signal transducer and activator of transcription 1
- **IIGP**: IFN-inducible GTPase
- **SCYA5**: RANTES. Chemotactic factor. Binds to CCR5
- **CMKBR5**: GPCR. Chemokine C-C receptor 5 (CCR5)

### Fold repression/Fold induction

- ≥10
- 6-4
- 2-1
- >2
- 4-6
- 10

**a**

**b**

**c**
### Cytokines, chemokines, growth factors

| Group       | Days | Function / Domain                                                                 |
|-------------|------|-----------------------------------------------------------------------------------|
| SCGF        | 1    | Secreted C-type lectin. Promotes growth of macrophage precursors                  |
| AIF1        | 3    | Role in macrophage activation and function                                         |
| IGF1        | 6    | Stimulates proliferation and differentiation of osteoblasts                       |
| SCYA7/MCP-3 | 1    | Chemotactic for monocytes. Induces the release of gelatinase b                     |
| PDGF-a      | 3    | Chemotactic factor for monocytes                                                   |
| GBP         | 6    | Galaptin (s-lectin) family. Autocrine growth inhibitor                              |
| SCYA5/RANTES| 1    | Stimulates proliferation and differentiation of osteoblasts                       |
| TGFBI/BIGH3 | 3    | Chemoattractant for monocytes. Binds to CCR5                                       |
| TNFSF9      | 6    | Kerato-epithelin. Inhibits bone nodules formation (in vitro)                       |
| SCYA8/MCP-2 | 1    | Involved in T lymphocytes activation                                               |
|             | 3    | Chemotactic for monocytes. Induced by IFN-γ, mitogens, IL-1                        |

#### Fold repression and Fold induction

- ≥10: >6
- ≥4: >4
- ≥2: >2
- 1:1: 1
- ≥1: 0
Fig. 4 b

| Receptors | Function / Ligand / Domains |
|-----------|----------------------------|
| FPR1      | Chemotactic factors        |
| EMR1      | GPCR                      |
| C3AR1     | Anaphylatoxin c3a.        |
| SDFR2     | Stromal cell derived factor receptor 2 |
| Clast3    | Member of the TNFRSF      |
| OPSR1     | GPCR. Neurosteroids,       |
| PTGER2    | Prostaglandin E receptor EP2 |
| IL11RA2   | Interleukin 11 receptor,  |
| LXR-a     | Orphan nuclear receptor,   |
| TTK/ESK   | Eph receptor PK family    |
| BP3/Bst1  | GPI-anchored membrane     |
| H2R       | Histamine. Acid secretion  |
| ADRA1A    | α1A-adrenergic receptor    |

Fold repression  Fold induction
| ≥10 | >6 | >4 | 2 1:1 | 2 >4 | >6 | ≥10 |

Group | E_ | F_ | G_ |
|------|----|----|----|
| Days | 1 3 6 | 1 3 6 | 1 3 6 |
**Fig. 4 c**

### Signaling molecules

| Group         | Function / Ligand / Domains                                                                 |
|---------------|---------------------------------------------------------------------------------------------|
| LIMK1         | PK with LIM-domain. Role in cytoskeletal changes, migration                                 |
| SEL1H         | Negative regulator of the Notch pathway                                                     |
| I4-3-3 z      | Regulator of CAMKII and PKC                                                                 |
| NM23-M6       | NDP kinase family. Role in the generation of multinucleated cells                           |
| DOK2          | Docking protein. Binds to Ras-GAP                                                            |
| DAB2          | Docking protein. P-Tyr interacting, SH3 binding                                              |
| PFTK1         | CDC2-related PK. Active in postmitotic/differentiated cells                                  |
| DMPK/DM15     | S/T PK (by homology). Modulator Ca \(^{2+}\) homeostasis                                    |
| PHLDA3        | Possible docking protein for receptors. PH-like domain                                        |
| PLFAP         | Proliferation-associated protein 1. Similar to ErbB3-binding EBP1                           |
| TRIP          | TRAF-interacting protein. Inhibits NF-kappaB, via TRAF2                                      |
| LAP18         | Stathmin. Relay (via phosphorylation) for various pathways                                   |
| NEK2          | S/T PK. Role in mitosis, meiosis                                                             |
| STK6          | S/T PK, centrosomal. Role in cell growth/chromosome segregation                             |
| ECT2          | Related to regulators of small GTP-binding proteins                                          |
| PLK           | S/T PK, nuclear. Role in cell division                                                       |
| RACGAP1       | Rac GTPase activating protein 1                                                              |
| VRK1          | S/T PK. Vaccinia related kinase 1                                                             |
| PLD1          | Phospholipase D1. Involved in various pathways, inflammation                                |
| RRAS          | NFκB target gene. Regulates the binding of integrins to their ligands                        |
| GRB10         | Docking protein for receptor PTK. SH2 domain                                                 |
| RALGDS/RGDS   | Guanine nucleotide dissociation stimulator. Effector of RRAS                                  |
| SOCS1/CISH1   | Inhibitor of IFN-γc-fms signaling                                                           |
| PIM1          | Inhibitor of IFN-signaling. IL-10 pathway                                                    |
| SOCS3         | PTK. Involved in integrin-signaling (cytoskeletal structure, motility)                      |
| FGR/SRC2      | PK. Co-repressor for homeodomain transcription factors                                       |

### Fold repression & Fold induction

- ≥10
- >6
- >4
- 1:1
- >2
- >4
- ≥10
Transcription factors and modulators

| Group | Function / Ligand / Domains |
|-------|-----------------------------|
| STAT3IP1 | Regulates STAT3 activation. Cytokine-signalling |
| CLOCK | bHLH TF |
| NAB2 | Corepressor for ZNF TFs (KROX20,24) |
| CIAO1 | Modulates activity of the WT1 tumor suppressor |
| ETL1 | ZNF transcriptional repressor |
| AEBP2 | |
| ZFP101 | Kruppel-associated box-containing ZNF protein |
| TFIIH1 | RNA polymerase II initiation factor b |
| c-myc | bHLH-Zip TF |
| LXRα | Orphan nuclear receptor. Interacts with RXR |
| GCMb | TF. Cell fate determination |
| CLIM-1b | LIM homeobox protein cofactor. Cell differentiation |
| HNF3/FOXM1 | Winged-helix transcription factor |
| JunB | |
| CSRP | ZNF/LIM nuclear protein. Cell differentiation |
| NFKB2 | NFκB2, 50 kDa DNA binding subunit. |
| ZFP63 | Kruppel-associated box-containing ZNF protein |
| TCF7 | HMG box-T-cell specific TF |
| RELB | Transactivation function of p50 NFκB |
| PHXR4 | bHLH TF |
| AHR | Inhibitor of ligand dependant transcriptional activator |
| MEF2B | Myocyte enhancer factor 2B. Muscle cell differentiation |
| HIPK2 | Nuclear PK. Co-repressor for homeodomain TF |
| E4BP4 | bZIP transcriptional regulator |
| LISCH7 | bHLH-Zip TF |

Fold repression vs Fold induction

- ≥10
- >6
- ≥4
- >2
- 1:1
- >4
- >6
- ≥10
| Factor | Gene Group Related to | Time (days) | Factor | Gene Group Related to |
|--------|----------------------|-------------|--------|----------------------|
| M-CSF  | RANK, Chemokines (-) Interleukins, Interferons (- -) | C | 1 | RANK, TRAF2A |
|        |                      | 3 |       | IL1a, IL11RA2, IL18, IGIFBP, GP130 |
|        |                      | 6 |       | IFNB1, IFNGR, STAT1, ILGP |
| RANKL  | Chemokines (-, - -)  | C | 1 | RANTES |
|        | Growth Factors (-, - -) | 3 |       | SCYA2,7 |
|        |                      | 6 |       | RANTES, MCP-2 |
|        | Receptors (-, - -, ...) | C | 1 | PDGF-a |
|        | Signaling (-, - -, ...) | 3 |       | IGF-1 |
|        | Transcription (-, - -, ...) | 6 |       | ETL1, CIAO1 |
|        |                      |       |       | ESK |
|        |                      |       |       | H2R, ADRA1A |
|        |                      |       |       | PTGER2, IL11RA2 |
|        |                      |       |       | DAB2, DMPK |
|        |                      |       |       | NEK2, PLD1 |
|        |                      |       |       | FGR |
|        |                      |       |       | CSRP, NFkB2 |
|        |                      |       |       | RELB, MEF2B |
Fig. 6

**Microarrays**

**qPCR**

Relative mRNA levels

| PDGF-α° | ScyA5/RANTES° | H2R° | CLOCK° | CSRP° |
|---------|---------------|------|--------|-------|
| ++ | ++ | ++ | ++ | ++ |
| + | + | + | + | + |
| - | - | - | - | - |
| 1 | 3 | 6 | Days | 1 | 3 | 6 | Days |

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Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFκB

David Cappellen, Ngoc-Hong Luong-Nguyen, Sandrine Bongiovanni, Olivier Grenet, Christoph Wanke and Mira Susa

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