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FAST TRACK

# Gene Array Identification of Osteoclast Genes: Differential Inhibition of Osteoclastogenesis by Cyclosporin A and Granulocyte Macrophage Colony Stimulating Factor

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**Abstract** Treatment of adherent peripheral blood mononuclear cells (PBMCs) with macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) stimulates the formation of multinucleate osteoclast-like cells. Treatment with M-CSF alone results in the formation of macrophage-like cells. Through the use of Atlas human cDNA expression arrays, genes regulated by RANKL were identified. Genes include numerous cytokines and cytokine receptors (RANTES and CSF2Rα), transcription factors (nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and GA binding protein transcription factor alpha (GABPα)), and ribosomal proteins (60S L17 and 40S S20). Real-time PCR analysis showed significant correlation ($R^2$ of 0.98 $P < 0.01$) with array data for all genes tested. Time courses showed differential activation patterns of transcription factors with early induction of FUSE binding protein 1 (FBP) and c-Jun, and later steady upregulation of NFATc1 and GABP by RANKL. Treatment with cyclosporin A, a known NFATc1 inhibitor, resulted in a blockade of osteoclast formation. The mononuclear cells resulting from high cyclosporin treatment (1,000 ng/ml) were cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP) positive but expression of calcitonin receptor (CTR) was downregulated by more than 30-fold. Constant exposure of M-CSF- and RANKL-treated cells to GM-CSF resulted in inhibition of osteoclast formation and the downregulation of CTSK and TRAP implicating the upregulation of CSF2R in a possible feedback inhibition of osteoclastogenesis. J. Cell. Biochem. 91: 303–315, 2004.


**Key words:** RANKL; NFAT; osteoclast; cyclosporin A; GM-CSF

Osteoclasts are multinuclear cells responsible for bone resorption [Yasuda et al., 1998]. Macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are the major cytokines involved in osteoclast differentiation [Matsuzaki et al., 1998; Quinn et al., 1998; Takahashi et al., 1999]. M-CSF is a soluble cytokine responsible for the proliferation, differentiation, activation, and survival of cells of the monocyte/macrophage lineage [Stanley et al., 1983]. RANKL is also known as osteoclast differentiation factor (ODF) or TNF-related activation-induced cytokine (TRANCE) and osteoprotegerin ligand (OPGL) [Yasuda et al., 1998]. RANKL is a membrane bound protein of the tumor necrosis factor (TNF) superfamily [Wong et al., 1997] that is expressed on the surface of osteoblasts and stromal cells where it is able to interact with RANK, a membrane bound protein present on the surface of osteoclast precursors [Yasuda et al., 1998]. RANK and RANKL are the principal mediators of interactions between

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osteoblast/stromal cells and osteoclast progenitors. This leads to a cascade of gene expression changes in osteoclast precursors resulting in osteoclast formation and activation.

Distinctive osteoclast gene markers include cathepsin K (CTSK) [Gelb et al., 1996; Gowen et al., 1999], tartrate-resistant acid phosphatase (TRAP) [Hayman et al., 1996], calcitonin receptor (CTR) [Nicholson et al., 1986], and V-ATPase [Chatterjee et al., 1992]. Other genes that may contribute to osteoclast differentiation and function have been catalogued through random cDNA sequencing [Sakai et al., 1995]. Several transcription factors have been linked to the developing osteoclast; microphthalmia-associated transcription factor (MITF) is involved in CTSK and TRAP expression [Luchin et al., 2000; Motyckova et al., 2001] and PU.1 is involved in the maturation of osteoclast/macrophage progenitors [Tondravi et al., 1997]. Although members of the TNF superfamily, including RANKL, activate NFκB, other unidentified transcription factors must be involved in osteoclastogenesis, as osteoclast differentiation is not an obligatory consequence of NFκB activation in all cell types. Recently, Ishida et al. [2002] and Takayanagi et al. [2002] presented gene array analysis of RANKL differentiated mouse osteoclasts. These authors focused on the role of transcription factor NFATc1 in osteoclast differentiation. NFAT is a multi-subunit transcription factor implicated in immune cell, muscle, and myocardium development [Horsley and Pavlath, 2002]. NFATc1 activation is regulated by the $Ca^{2+}$/calmodulin-dependent protein phosphatase calcineurin [Northrop et al., 1994], a downstream target of increased intracellular calcium levels. The binding of RANKL to RANK has also been implicated in increased cytosolic $Ca^{2+}$ levels of osteoclast-like cells isolated from osteosarcoma tumors [Myers et al., 1999]. In this study, we verify the induction of NFATc1 by RANKL in human osteoclasts differentiating in vitro, and also add substantially to the list of transcription factors induced during osteoclast differentiation. Furthermore, we show that cyclosporin A inhibits fusion to multinuclear giant cells and represses calcitonin receptor but does not affect the expression of CTSK nor tartrate-resistant acid phosphatase. In addition, we demonstrate that the alpha subunit of GM-CSF receptor is upregulated by RANKL, and that exogenous GM-CSF represses osteoclast-related gene expression and differentiation.

## MATERIALS AND METHODS

### Preparation of Osteoclast and Macrophage-Like Cells

Cells were cultured in minimal essentials medium (MEM) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and 100 μg/ml penicillin-streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from healthy donors under a protocol approved by Barwon Health Research and Ethics Committee. PBMCs were isolated from buffy coats using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) as per Nicholson et al. [2000]. PBMCs were plated at $10^8$ cells per 75 $cm^2$ flask in 10 ml MEM and allowed to adhere to the flask for 2 h at 37°C, 5% $CO_2$. Non-adherent cells were removed from the flask by washing twice with MEM. Adherent PBMCs were treated with 40 ng/ml RANKL and 25 ng/ml M-CSF (Peprotech, Rocky Hill, NJ) to produce osteoclasts, while 25 ng/ml of M-CSF alone was used to induce macrophages. Medium supplemented with M-CSF, or M-CSF and RANKL was replaced every 3–7 days for up to 3 weeks. Six separate preparations were made, at different times, from six different donors. The first three preparations were used to verify the differentiation of osteoclasts using microscopy and quantitative real-time PCR of osteoclast marker genes. The fourth preparation was used for array hybridizations and the final two preparations were used for real-time quantitative PCR analysis of novel genes identified through array analysis. Another three preparations were made for both cyclosporin A (Sigma-Aldrich, St. Louis, MO) treatment and GM-CSF treatment. Cyclosporin A treatment was performed on standard M-CSF- and RANKL-treated cells at concentrations between 10 and 1,000 ng/ml as per Ishida et al. [2002]. TRAP staining was performed using the Sigma leukocyte acid phosphatase staining kit. GM-CSF-treated cells were grown in the presence of M-CSF and RANKL (as above) and 25 ng/ml of GM-CSF (Peprotech, Rocky Hill, NJ) to inhibit osteoclast formation.

### RNA Isolation and Probe Preparation

At the end of 3 weeks, the cells were lysed, and the RNA harvested using 4 M guanidium isothiocyanate, 1% lauryl sarcosine lysis, followed by ultracentrifugation over a 5.7 M cesium chloride, 100 mM EDTA cushion in a Beckman

SW41 rotor at 27,000 rpm for 16 h [Sambrook et al., 1989]. The RNA was dissolved in 1% SDS in TE buffer (pH 7.5), extracted with chloroform then ethanol precipitated, washed in 75% ethanol (0.8 ml/50–100 μg of RNA), then dissolved in DEPC-treated water, and quantitated by spectrophotometry.

cDNA was produced using MMLV reverse transcriptase (RT, BD Biosciences, Franklin Lakes, NJ). For every 5 μg of total RNA, 1 μg 19mer dT primer mix was added. The mixture was then heated to 70°C for 5 min to remove any secondary structure in the RNA, then placed on ice and 4 μl of five times RT reaction buffer (50 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 500 μM dNTP mix, 5 U of Rnasin, 25 U of RT, and water was added to a total volume of 20 μl. The reaction was incubated for 1 h at 42°C, before heat inactivation at 75°C for 5 min. Probe was amplified by adding 5 μg of cDNA to a reaction mix containing 4 μl of five times RT reaction buffer, 2 μl of each CDS primer (BD Biosciences, NJ), 5 pmoles of arbitrary 13-mer primer (AAGCTTTTACCGC), 10 pmoles of random primers, 1 μl 100 μM dNTPs (no C), 0.1 μl 100 μM $^{32}$PdCTP (Amersham Biosciences, Buckinghamshire, England), 6.7 μl $H_2O$, and 1 U of *Taq* polymerase (Life Technologies). The reaction was denatured at 94°C for 3 min, primed at 42°C for 2 min, and extended at 65°C for 1 min. Thermocycling was for 20 cycles, each consisting of a 30 s denaturation step at 94°C, followed by a 42°C priming step for 1 min and a 65°C extension step for 1 min. Unincorporated radioactive nucleotide was removed using Sephadex chromatography (G-50 Nick-columns, Amersham Biosciences, Buckinghamshire, England).

### Array Hybridization

Hybridization was under standard conditions (6× SSPE, 1× Denhardt's solution, 50% formamide, 10 μg/ml herring sperm DNA) with 16 h pre-hybridization and 16 h hybridization at 42°C. Filters were washed at 68°C through a traditional series of decreasing ionic strength washes (2× SSC, 2× SSC 0.1%SDS, then 0.6× SSC 0.5% SDS). Radioactivity was visualized using a Bio-Rad personal FX phosphor imager and Kodak phosphor storage screens. Data were quantified using Quantity One software (Bio-Rad, Hercules, CA), and analyzed using Microsoft Excel and the SPSS statistics package. The absolute counts per spot minus adjacent background were used as a measure of hybridization and the ratio of such counts from the two treatments taken as fold change in expression.

### Real-Time Quantitative PCR (Q-PCR)

Total RNA for Q-PCR was isolated as described above at 3-day, 1-week, and 3-week time points and converted into cDNA using Superscript II RT (Invitrogen, Carlsbad, CA) and oligo dT primer. PCRs were performed using SYBR Green Supermix (Bio-Rad, Hercules, CA). PCR primers were designed using the assistance of the BLAT server (genome.usca.edu) to identify, where possible, reasonably short introns near the 3′-end of the gene. PCR reactions were optimized on genomic DNA and cDNA in order to verify the presence of the short intron in genomic DNAs. All PCR reactions were optimized for an annealing temperature of 55°C by varying magnesium or primer concentration. Primer concentrations were between 100 and 500 nM in a final volume of 20 μl. The reactions were then cycled as follows: step 1, 94°C for 2 min; step 2, 94°C for 30 s, 55°C for 45 s, 72°C for 1 min (45 cycles); step 3, melt curve analysis from 55 to 95°C in 0.5°C increments. Each Q-PCR reaction was set up using 50 ng cDNA per reaction, along with a genomic DNA control (100 ng per reaction), and a DNA-free water control. PCR were performed using the Bio-Rad iCycler iQ system. All PCR reactions were tested for linearity of response using standard curves of between 10 and $10^9$ copies of cDNA and PCR product were analyzed by polyacrylamide gel electrophoresis (PAGE) to verify the specificity of amplification and exclude the possibility of genomic DNA contamination. Primers and product characteristics are listed in Table I. Q-PCR analysis on housekeeping genes β-actin and 18S Ribosomal RNA showed no significant difference in cycle threshold with identical quantities of input cDNA, indicating that either of these genes was a reasonable housekeeping control for real-time quantitative PCR (data not shown). Subsequently, 18S was chosen as the standard control. The cycle threshold (Ct) of gene expression in different RNA samples acquired through the amplification curves was used to analyze the relative expression (fold regulation). To calculate the fold difference in gene expression, the difference in Ct in each RNA sample is calculated using the equation: $\text{Fold} = 2^{\Delta\Delta Ct}$ as per Livak and Schmittgen

**TABLE I. Primers Used for Real-Time Quantitative PCR Are Listed in 5′ to 3′ Orientation With the Sizes of Amplicons in Base Pairs From cDNAs and From Genomic DNA**

| Gene | Forward primer | Reverse primer | Genomic | cDNA |
|---|---|---|---|---|
| 18S | CTTAGAGGGACAAGTGGCG | ACGCTGAGCCAGTCAGTGTA | 106 | 106 |
| c-Jun | GCGTTAGCATGAGTTGGCAC | CGCATGAGGAACCGCATCGC | 201 | 201 |
| Calmodulin 1 | GGCATTCCGAGTCTTTGACAA | CCGTCTCCATCAATATCTGCT | 511 | 140 |
| Cathepsin K | TGAGGCTTCTCTTGGTGTCCATAC | AAAGGGTGTCATTACTGCGGG | 219 | 134 |
| CD44 | TGGCACCCGCTATGTCCAG | GTAGCAGGGATTCTGTCTG | 212 | 212 |
| CDK7 | ATGGCTCTGGACGTGAAGTC | CTTAATGGCGACAATTTGGTTG | 474 | 122 |
| CSF2Rα | GGCACGAGGCGAGAGAAGA | ACGCAAACATCGCCGCTTCT | ND | 112 |
| CTR | TGGTGCCAACCACTATCCATGC | CACAAGTGCCGCCATGACAG | 201 | 118 |
| FBP1 | CATGGCGATGGACCGGGA | AGGTTTGTCAGCACCAGTGT | 263 | 167 |
| GABPα | AAAGAGCGCCGAGGATTTCAG | CCAAGAAATGCAGTCTCGAG | 357 | 133 |
| GABPβ | CCCAGAGAGTCCTGACACT | TCTGAAGAATTGGACAATGG | 505 | 188 |
| NF90 | AGGCCTACGCTGCTCTTGCT | GCCGAAGCCAGGGTTATGTG | 234 | 142 |
| NFATc1 | GCATCACAGGGAAGACCGTGTC | GAAGTTCAATGTCGGAGTTTCTGAG | 2100 | 152 |
| TRAP | GACCACCTTGGCAATGTCTCTG | TGGCTGAGGAAGTCATCTGAGTTG | 301 | 176 |
| ZFP33a | AAGATGTGACTGTGGGCTTCA | GATCACCTCTGGTTTGTGAAC | 413 | 139 |

[2001]. Correlation of Q-PCR results to array results was performed using SPSS statistical analysis software.

## RESULTS

### Microscopy and Molecular Characterization of PBMC Differentiation

Verification of the model was performed by microscopy of the differentiating cells. Figure 1A,B shows a stain for TRAP activity at 3-week post-treatment (M-CSF treatment in Fig. 1A, M-CSF and RANKL treatment in Fig. 1B). The absolute expression of CTSK and TRAP were also investigated using quantitative real-time PCR. mRNA was harvested from the treated PBMCs at 3-day, 1-week, and 3-week time points during the 3-week differentiation time course and reverse transcribed into cDNA. Absolute expression of CTSK increased across the time course in RANKL and M-CSF compared with cells treated with M-CSF alone (Fig. 1C). TRAP also showed a steady increase in expression across the 3-week time course in M-CSF- and RANKL-treated cells (Fig. 1D).

### Expression Profile Using Atlas 1.2 Array Set

The 3-week time point was chosen for array analysis since, in this human system, the greatest difference in morphology and expression of TRAP and CTSK was observed at this time point. Changes in gene expression, resulting from 3 weeks of RANKL treatment of PBMCs, were revealed by hybridization with the Atlas 1.2 human array system. The system is a group of three nylon arrays, each containing 1,176 genes representing over 3,500 different genes. The results of the three combined arrays showed 128 genes regulated greater than fivefold with 35 genes regulated greater than tenfold. These genes have been broken up into related functions or families (see Tables II–IV).

The Atlas array showed regulation within several families of genes. Nine of the regulated genes were transcription factors or transcription factor subunits. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), was present on both Atlas 1.2 I and II arrays, both showing greater than 16-fold upregulation. Early growth response factor 1 (EGR1) was upregulated to a similar level to NFATc1. Zinc finger protein 33a (ZFP33a) showed the highest regulation of the transcription modulators with 115-fold downregulation after treatment with RANKL (Table II).

Fourteen cytokine-related and cell surface proteins were also regulated more than fivefold and present with high signal on the array (Table III). The GM-CSF receptor (or colony stimulating factor 2 receptor alpha, CSF2Rα) and HLA-C show the greatest regulation in this group, more than 40-fold up for CSF2Rα and more than 10-fold down for HLA-C. Other CSF-related genes (CSF3R and IL-5) are also upregulated by RANKL. Three TNF-related genes were upregulated by RANKL with CD70, at 14-fold, being the highest induced. Sixteen other genes show regulation of tenfold or more on the array (Table IV). These include cell cycle related genes, activators of protein kinases, ribosomal proteins, DNA repair proteins, and genes involved in cell metabolism.

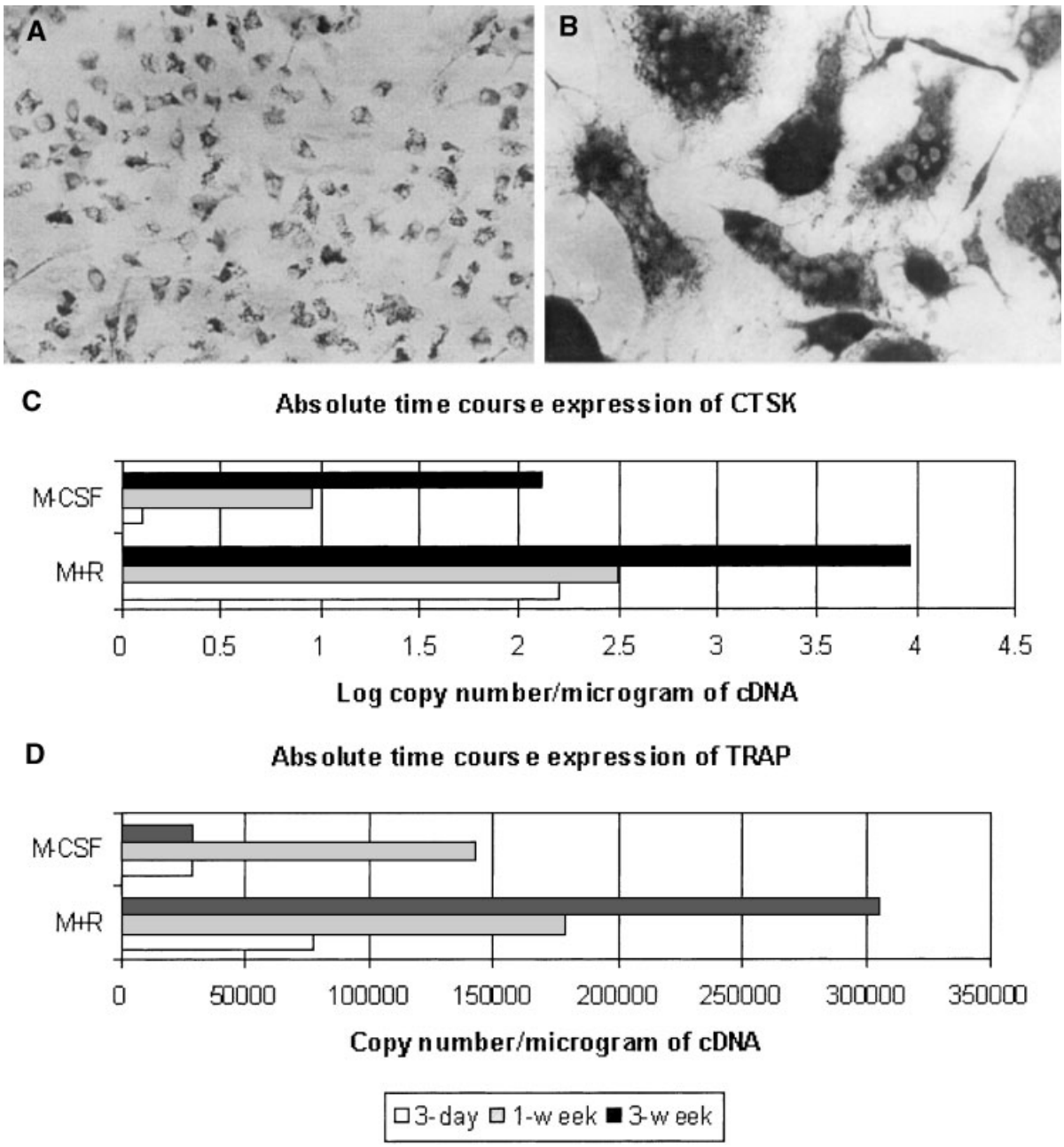


**Fig. 1.** **A**: TRAP-stained 3-week cultures of M-CSF-treated cells. **B**: TRAP-stained 3-week cultures of M-CSF- and RANKL-treated cells. **C**: Absolute expression of cathepsin K (CTSK) at 3-day, 1-week, and 3-week time points. **D**: Absolute expression of TRAP across the 3-week time course. (M + R: M-CSF and RANKL).

### Q-PCR Verification of Array Data

To assess the validity of the regulation of gene expression shown by the arrays, Q-PCR was performed on ten genes using RNA derived from independent cell differentiations, unrelated to those used for the array, and derived from blood obtained from two different individuals. Eight genes that were upregulated, one gene that was not regulated, and one gene that was strongly downregulated by RANKL were analyzed for fold difference by Q-PCR (Table V). All ten genes showed comparable regulation when Q-PCR results were compared to the array result (correlation coefficient 0.98). Seven induced genes were determined in the two different cDNA

**TABLE II. Modulators of Gene Transcription Regulated by RANKL**

| Gene name | Genbank | OC | MAC | Fold |
|---|---|---|---|---|
| Nuclear factor of activated T-cells, cytoplasmic 1 (1.2 I) | U08015 | 20668 | 886 | 23.3 |
| Far upstream element (FUSE) binding protein 1 (FBP) | U05040 | 5455 | 315 | 17.3 |
| Nuclear factor of activated T-cells, 90 kD (ILF-3) | U10324 | 7803 | 476 | 16.4 |
| Nuclear factor of activated T-cells, cytoplasmic 1 (1.2 II) | U08015 | 110231 | 6824 | 16.2 |
| GA-BP transcription factor, alpha subunit 60 kD | D13318 | 6063 | 392 | 15.5 |
| Early growth response 1 | M62829 | 12812 | 962 | 13.3 |
| LIM homeobox protein 1 | U14755 | 40747 | 3263 | 12.5 |
| REL-B | M83221 | 627 | 5995 | −11.4 |
| Deltex | AF053700 | 3072 | 47495 | −15.4 |
| Zinc finger protein 33A (ZNF KOX31) | X68687 | 347 | 39858 | −115 |

Table contains gene name, Genbank accession number, phosphor image quantitation for osteoclast and macrophage probes, and change in gene expression of osteoclast relative to macrophage. Data are ranked according to fold difference. (Nuclear factor of activated T-cells, cytoplasmic 1, was present on both 1.2 I and II array as indicated.)

preparations; c-Jun, ZFP33a, CD44, NF90, calmodulin 1, GABP-α, CSF2Rα, and NFATc1 (Table V). The average difference in the estimated ΔΔCt value over the seven genes was 0.3 cycles, verifying reproducibility in different cell preparations. These data, coupled with the similarity in array and Q-PCR data, indicate a remarkable reproducibility in the differentiation model and a high correlation between array data and Q-PCR data in the ten genes analyzed, suggesting that the array data for other genes are reliable.

The following transcription factors showed regulation at the three week time point by both array analysis and Q-PCR: NFATc1, NFAT90 (ILF3), ZFP33a, FBP, GABPα, and c-Jun. GABPβ was analyzed only by Q-PCR and was upregulated (Fig. 2). The time course data suggest different kinetics of induction. In particular, the kinetics of NFATc1 appears similar to GABPα and beta and NF90, with later increasing expression, while FBP and c-Jun expression rise earlier in the treatment period then decrease.

### Effects of NFAT Inhibitors on M-CSF and RANKL-Treated Cells

Cyclosporin A is an inhibitor of NFATc1, although effects on other proteins, such as p38 and/or JNK kinases, are known [Matsuda et al., 2000]. Treatment of cells with cyclosporin A (concentrations between 10 and 1,000 ng/ml) over 3-week culture resulted in atypical cell morphology. Rather than TRAP positive multinucleated giant cells, the cells decreased in size across the dose response until at 1,000 ng/ml, the majority of cells were mono-nucleated and TRAP positive (Fig. 3, Table VI). These data are similar to the observations of Ishida et al. in the mouse RAW264.7 cell model, where cyclosporin A repressed the formation of multinucleated cells, resulting in a majority of mononuclear cells that were TRAP positive. We considered that NFATc1 might be a late transcription

**TABLE III. Cell Surface Proteins and Cytokine-Related Genes**

| Gene name | Genbank | OC | MAC | Fold |
|---|---|---|---|---|
| GM-CSF receptor (CSF2R) α, low-affinity | 17648 | 11180 | 259 | 43.2 |
| Cadherin 5, VE-cadherin (vascular epithelium) | X79981 | 57366 | 3568 | 16.1 |
| Adenosine A1 receptor | S56143 | 3322 | 220 | 15.1 |
| CD44 antigen | M59040 | 62050 | 4335 | 14.3 |
| TNF (ligand) superfamily, member 7 (CD70) | L08096 | 2966 | 203 | 14.6 |
| G-CSF receptor (CSF3R) | M59818 | 5347 | 452 | 11.8 |
| Small inducible cytokine A5 (RANTES) | M21121 | 3840 | 340 | 11.3 |
| Chemokine (C-C motif) receptor 2 | U03882 | 5231 | 484 | 10.8 |
| Integrin, alpha L (antigen CD11A (p180)) | Y00796 | 5913 | 594 | 10.0 |
| TNF superfamily, member 1 (TNF-β) | D12614 | 1803 | 207 | 8.7 |
| Interleukin 5 (CSF-eosinophil) | X04688 | 4540 | 582 | 7.8 |
| Integrin, alpha 3 (antigen CD49C) | M59911 | 52757 | 8462 | 6.2 |
| TNF (TNF superfamily, member 2) (TNF-α) | X01394 | 1007 | 197 | 5.1 |
| Histocompatibility complex class I C (HLAC) | M11886 | 287 | 3420 | −11.9 |

Table contains gene name, Genbank accession, phosphor image data, and fold relative expression difference in osteoclast relative to macrophages. Data are ranked according to fold difference in expression. Genes with <10-fold regulation are included because of high relative levels of phosphor image signal.

**TABLE IV. Other Genes Regulated by RANKL**

| Gene name | Genbank | OC | MAC | Fold |
|---|---|---|---|---|
| Cyclin-dependent kinase 7 | L20320 | 5600 | 186 | 30.1 |
| Calmodulin 1 (phosphorylase kinase, delta) | M27319 | 15011 | 556 | 27 |
| 40S ribosomal protein S20 (RPS20) | L06498 | 4047 | 205 | 19.8 |
| A kinase (PRKA) anchor protein 5 | M90359 | 38291 | 2650 | 14.4 |
| Discs, large (*Drosophila*) homolog 1 | U13897 | 9183 | 720 | 12.8 |
| Potassium voltage-gated channel, shaker-related subfamily 2 | L02752 | 17310 | 1453 | 11.9 |
| Tuberous sclerosis 2 | X75621 | 17099 | 1469 | 11.6 |
| Thrombospondin 2 | L12350 | 41931 | 3807 | 11 |
| Caspase 10, apoptosis-related cysteine protease | U60519 | 6410 | 597 | 10.7 |
| PCTAIRE protein kinase 1 | X66363 | 9545 | 920 | 10.4 |
| Protein arginine N-methyltransferase 3 | AF059531 | 4028 | 43987 | −10.9 |
| Alpha-synuclein | L08850 | 1631 | 26046 | −15.9 |
| X-ray repair complementing defective repair 1 | M36089 | 329 | 7228 | −22.6 |
| 60S ribosomal protein L17 (RPL17) | X53777 | 1168 | 112507 | −96.1 |
| Ornithine decarboxylase antizyme (ODC-AZ) | D78361 | 163 | 24193 | −147 |
| Myosin light chain, non-muscle isoform (MLC3NM) | M22919 | 90 | 16863 | −187 |

Table contains gene name, Genbank accession, phosphor image data, and fold relative expression difference in osteoclast relative to macrophage. Data are ranked according to fold difference in expression.

factor in the osteoclast differentiation cascade, involved in multi-nucleation. Dose response data show that cyclosporin A uncouples the regulation of three major osteoclast markers, CTSK, TRAP, and calcitonin receptor. There was a profound and dose-dependent downregulation of calcitonin receptor in cells treated with cyclosporin A (Fig. 4). Averaged across the three experiments and in concentrations that were maximal (>200 ng/ml), the repression of CTR was $5.9 \pm 0.4$ cycles ($\pm$SE, $P = 4 \times 10^{-8}$). In contrast, CTSK and TRAP were unaffected by cyclosporin A ($P = 0.99$). Less than one cycle difference in CTSK and TRAP expression was observed across the dose response. These data suggest different mechanisms of regulation apply to CTSK, TRAP, and calcitonin receptor, where NFATc1 is necessary for calcitonin receptor but not CTSK or TRAP expression.

**TABLE V. Comparison of Atlas Array Analysis With Quantitative Real-Time PCR in Replicate cDNA Derived From Different Cell Preparations**

| Gene name | Array | Q-PCR 1 | Q-PCR 2 |
|---|---|---|---|
| c-Jun | 1.45 | 1.52 | 2.3 |
| Calmodulin 1 | 27.0 | 22.1 | 20.5 |
| CD44 | 14.3 | 9.9 | 8.9 |
| CDK7 | 30.1 | 29.2 | — |
| CSF2Rα | 43.2 | 21.7 | 21.2 |
| FBP | 17.3 | — | 8.6 |
| GABPα | 15.5 | 24.3 | 22.3 |
| NF90 | 16.4 | 12.7 | 11.0 |
| NFATc1 | 23.3, 16.2 | 27.8 | 24.7 |
| ZFP 33a | −115 | −455 | −670 |

### Effects of GM-CSF on M-CSF and RANKL-Treated Cells

The 20-fold induction of GM-CSF receptor (CSF2Rα) in osteoclasts led us to further investigate the role of GM-CSF in osteoclastogenesis. We tested the preliminary hypothesis that GM-CSF receptor sensitizes the differentiation process to feedback inhibition leading to suppressed osteoclast formation. We can also determine if GM-CSF blocks osteoclast differentiation at an earlier or later stage than the cyclosporin blockade of NFATc1 described above. For instance, cyclosporin permits expression of TRAP and CTSK but blocks multinucleation. Continuous exposure of RANKL and M-CSF-treated cells to GM-CSF blocked osteoclast differentiation in a dose-dependent manner (data not shown). The expression of characteristic genes was investigated at high dose GM-CSF treatment, where visible osteoclasts were absent from culture vessels. Examination of CTSK and TRAP expression was performed showing marked downregulation in cells treated with GM-CSF, RANKL, and M-CSF compared with cells treated with RANKL and M-CSF, where characteristic osteoclast-like cells were present (Fig. 5).

## DISCUSSION

In this study, we add substantially to the list of genes regulated by RANKL in the osteoclast developmental pathway, verifying ten of these genes through Q-PCR, with striking concordance of Q-PCR and array result. Some of these

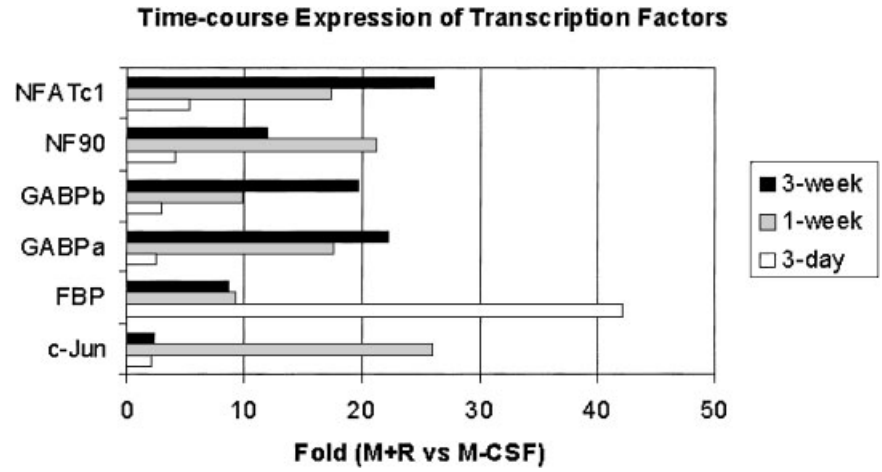


**Fig. 2.** Changes in fold regulations of transcription factors at various time points of osteoclasts versus macrophages using different cDNA over time periods of 3 days, 1 week, and 3 weeks.

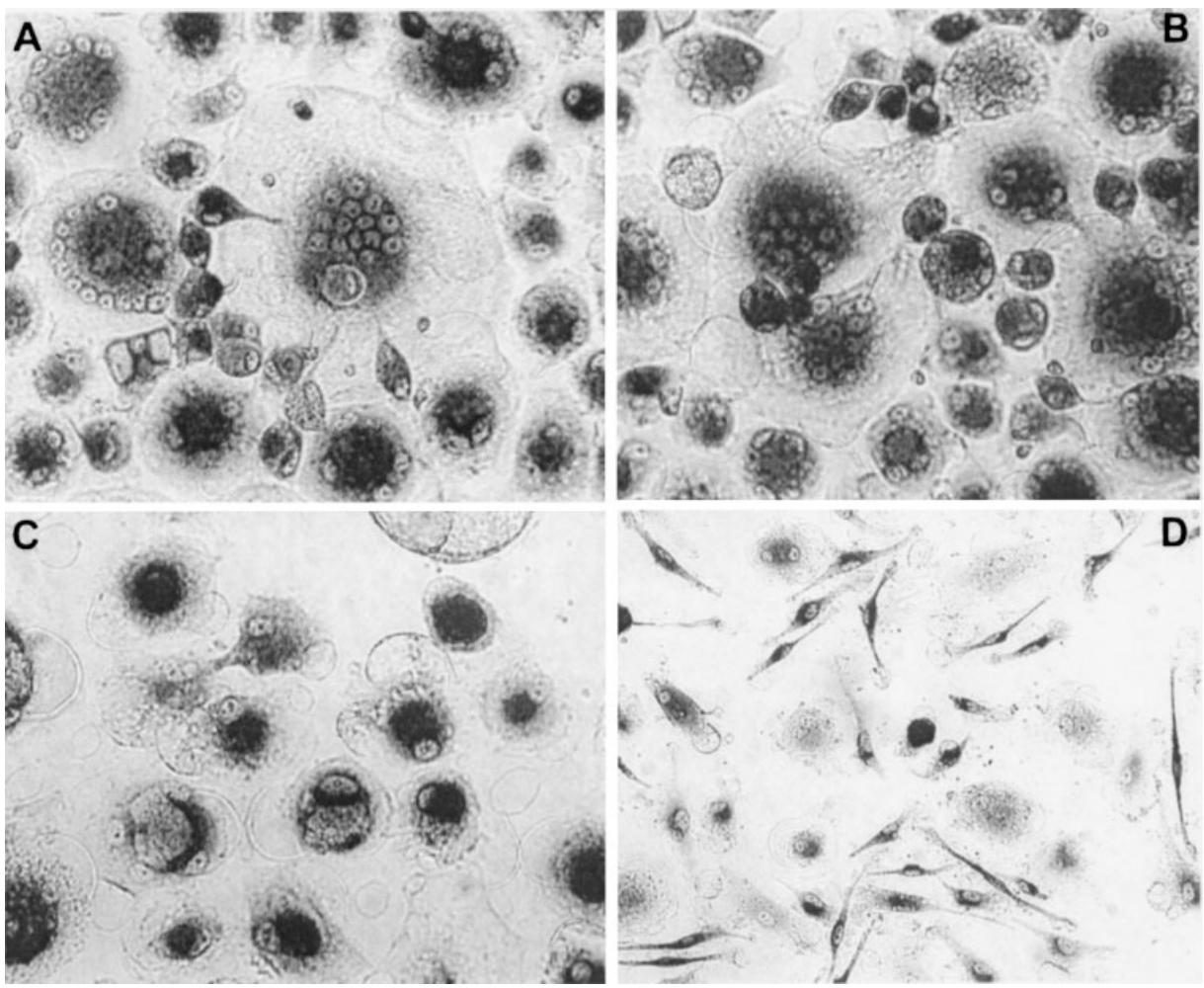


**Fig. 3.** Inhibition of osteoclast formation by cyclosporin A. **A**: M-CSF and RANKL cells treated with 10 ng/ml cyclosporin A. **B**: M-CSF and RANKL cells treated with 50 ng/ml cyclosporin A. **C**: M-CSF and RANKL cells treated with 250 ng/ml cyclosporin A. **D**: M-CSF and RANKL cells treated with 1,000 ng/ml cyclosporin A.

**TABLE VI. Effect of Cyclosporin A Treatment on the Multinucleation (MN) of M-CSF and RANKL-Treated Cells**

| Cyclosporin A (ng/ml) | Total cells | MNs | %MN | SE |
|---|---|---|---|---|
| 10 | 165 | 80 | 48.48 | 0.039 |
| 50 | 179 | 68 | 37.99 | 0.036 |
| 250 | 111 | 18 | 16.2 | 0.035 |
| 1,000 | 135 | 5 | 3.70 | 0.016 |

genes suggest immediate hypotheses concerning cytokine regulation of the differentiation pathway, others suggest the involvement of known transcription factors, not yet implicated in osteoclast functions, while the role of other genes remains enigmatic. In addition, we have identified genes that are actively switched off in the osteoclast differentiation pathway in comparison to the default macrophage pathway. Surprisingly, few of the genes reported here were described in other available array data on the osteoclast differentiation model that derived from mouse bone marrow cells and RAW264.7 [Cappellen et al., 2002; Ishida et al., 2002; Takayanagi et al., 2002]; however, this difference may result from the particular gene lists used to construct the respective arrays.

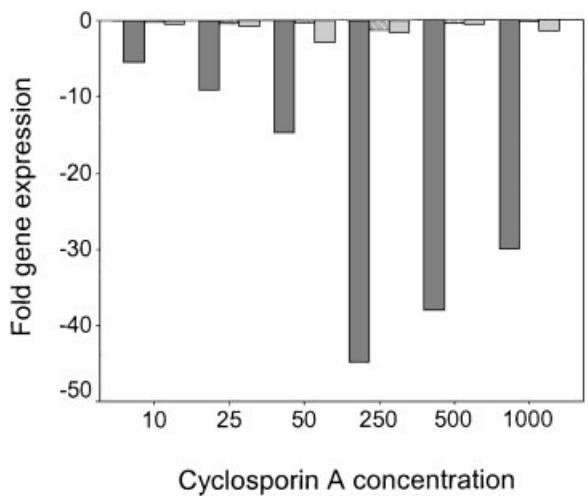


**Fig. 4.** Graphical representation of gene expression by cyclosporin A, as relative fold compared to M-CSF- and RANKL-treated control cells. Fold gene expression is $2^{\Delta\Delta Ct}$ relative to 18S with respect to the dose of cyclosporin A (0–1,000 ng/ml). Hatched boxes are CTSK (cross hatching) and TRAP (vertical hatching) showing no repression. Calcitonin receptor; unhatched columns showing profound repression.

This study adds to the list of genes expressed in osteoclasts and extends the finding of induction of NFATc1 in mouse osteoclasts to the human model system. However, we show that NFATc1 is just one of a number of transcription factors in human osteoclasts that are similarly induced by RANKL and that these factors follow different induction profiles. Furthermore, we use array data to derive testable hypotheses on two of the highly induced genes, demonstrating that blockade of osteoclast differentiation by cyclosporin A and GM-CSF occurs at different points.

Previously, a limited number of transcription factors were known to be involved in osteoclast differentiation. Of the previously known transcription factors, PU.1 acts at the root of the myeloid differentiation pathway that leads to mononuclear phagocytes and macrophages. PU.1 knockout animals lack macrophages and osteoclasts [Tondravi et al., 1997]. NFATc1 has recently been identified as a downstream target of RANKL [Ishida et al., 2002; Takayanagi et al., 2002].

Time course data illustrate that the temporal profiles of six transcription factors differ throughout osteoclastogenesis (Fig. 2). FBP is a factor that interacts with the far upstream element (FUSE) found 1.5-kb upstream of the proto-oncogene c-myc [He et al., 2000]. FBP was highly upregulated by RANKL at 3 days (42-fold) decreasing to less than tenfold by 3 weeks. The regulation of FBP indicates involvement in early osteoclast differentiation. GABP is a ubiquitously expressed transcription factor composed of two subunits (GABP$\alpha$ and GABP$\beta$) and is involved in the regulation of mitochondrial transcription factor A [Gugneja et al., 1995; Batchelor et al., 1998]. Q-PCR time course of the two GABP subunits showed similar regulation across time, peaking at 3 weeks (22.3-fold for $\alpha$ and 19.7-fold for $\beta$). The jun proto-oncogene (c-Jun) is known to be necessary for regulating cell cycle and hence, cell development [Takayanagi et al., 2002]. The time course expression of c-Jun showed high regulation by RANKL at 1-week time-point (26-fold) with low regulation at 3-day and 3-week time points. NFATc1 and NF90 showed similar profiles to that of GABP. NF90 was originally cloned as a component of the NFAT complex, while more recent data confirm NF90 as a transcriptional regulator but through a capacity to bind to RNA rather than DNA [Reichman et al., 2002]. The role of this protein in osteoclast

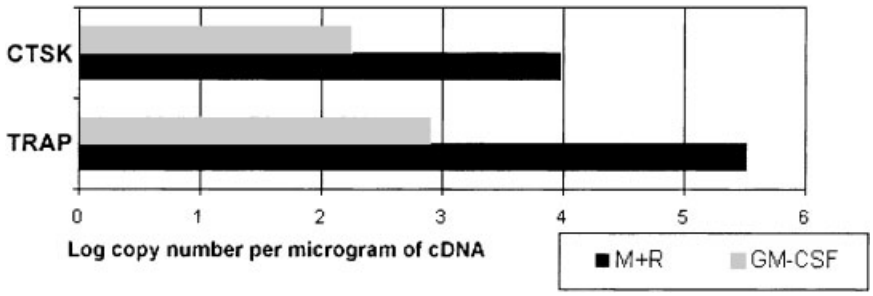


**Fig. 5.** Inhibition of CTSK and TRAP by GM-CSF in the continuous presence of M-CSF and RANKL (gray columns GM-CSF on the figure) compared with M-CSF and RANKL alone treated cells (black columns) at 3 weeks. (M + R: M-CSF and RANKL).

function remains to be determined. Array data (Table III) showed that EGR-1, a zinc finger transcription factor, was induced to an extent similar to NFATc1. Although we did not study this factor in time courses, recent data show that ERG-1 can heterodimerize with NFATc1 to regulate the IL-2 and TNF-α promoters in Jurkat cells [Decker et al., 2003]. IL-2 was not detected above background in the array (not shown), while TNF-α was 5.1-fold upregulated in the osteoclast preparation relative to macrophage, but this was from low background counts (Table III). Further work is needed on the potential interaction of EGR-1 and NFATc1 in osteoclasts.

Based on our observations, we concurred with Ishida et al. [2002] and Takayanagi et al. [2002] regarding the importance of NFAT in osteoclastogenesis. We showed NFAT upregulation across the differentiation time course and through inhibition of multinucleation by cyclosporin A, we confirmed the role of NFAT in human osteoclast formation (Figs. 2 and 3, Table VI). In human osteoclastogenesis, cyclosporin exposure results in mononuclear TRAP positive cells (Fig. 3). Cyclosporin A uncouples the expression of CTSK and TRAP from that of calcitonin receptor, where CTSK and TRAP appear unaffected by cyclosporin A, and calcitonin receptor shows profound downregulation by cyclosporin A treatment (Fig. 4). Time course data (Fig. 1) show that CTSK and TRAP are early-induced genes. These data are consistent with NFATc1 being involved in late events in osteoclastogenesis and in the expression of calcitonin receptor but not CTSK or TRAP. Interestingly, Anusaksathien et al. [2001] analyzed the CTR promoter revealing the P3 promoter of CTR to be active in osteoclasts. The P3 promoter of CTR was seen to contain multiple putative AP1/NFAT binding sites, providing further evidence of a link between NFAT and CTR expression in osteoclasts [Anusaksathien et al., 2001].

Table III shows fourteen genes that are cell surface molecules or cytokine-related genes regulated more than fivefold. Several of these genes have been implicated in some way to osteoclastogenesis or osteoclast function including GM-CSF, G-CSF receptor (CSF3R), TNFα, CD44, and RANTES. The most profoundly regulated receptor was GM-CSF receptor, although Q-PCR analysis showed 22-fold instead of 43-fold change in expression. To our knowledge, the upregulation of GM-CSF receptor by RANKL has not been reported previously, although GM-CSF is reported to inhibit RANKL-induced osteoclast formation [Miyamoto et al., 2001]. This suggests that the osteoclast may be sensitized to feedback inhibition by GM-CSF. We also found that GM-CSF treatment inhibited the upregulation of TRAP and CTSK by RANKL (Fig. 5). Repression of osteoclastogenesis by GM-CSF was of different character to that mediated by cyclosporin through NFATc1. Whereas cyclosporin-treated cells still expressed CTSK and TRAP, GM-CSF-treated cells showed no osteoclast characteristics at all, and all assayed genes related to osteoclast function were repressed. GM-CSF apparently overrides RANKL at the earliest stages of osteoclast differentiation.

The array reveals opportunities many more opportunities for further work, beyond the scope of this study; some of these will be discussed. G-CSF has been shown to increase osteoclast resorption leading to decreased bone mass in humans with congenital neutropenia

[Sekhar et al., 2001] and normal rats [Soshi et al., 1996] exposed to G-CSF and in transgenic mice overexpressing G-CSF [Takahashi et al., 1996]. In the case of osteopenia, resulting from prolonged treatment with G-CSF of human congenital neutropenia, bone mass can be recovered with bisphosphonate treatment [Sekhar et al., 2001]. The upregulation of the G-CSF receptor by 11.8-fold (Table III) with RANKL treatment may be physiologically meaningful to osteoclast function and requires further work.

CD44 is a cell surface glycoprotein known to bind osteopontin, an important non-collagen protein present in the bone matrix that aids in osteoclast attachment [Weber et al., 1996]. Antibodies to CD44 inhibited in vitro osteoclast-like cell formation from mouse bone marrow precursors [Kania et al., 1997].

In keeping with our observations, small inducible cytokine A5 (RANTES) was upregulated during RANKL-stimulated differentiation of mouse bone marrow and RAW 264.7 cells into osteoclasts [Cappellen et al., 2002; Ishida et al., 2002; Takayanagi et al., 2002]. RANTES was upregulated by more than tenfold by RANKL in the mouse microarray experiment by Cappellen et al. [2002], similar to our observation of 11.3-fold regulation (Table III). RANTES acts as a chemoattractant, possibly allowing osteoclast-like cells to attract other cells to specific locations. Surprisingly, RANTES and NFATc1 are the only genes observed in common between the mouse experiments of Cappellen et al. [2002], Ishida et al. [2002], and Takayanagi et al. [2002] and our human work. While it seems very unlikely that mouse and human will differ substantially, greater overlap might have been expected. Clearly, a more comprehensive array analysis of both human and mouse systems is required to finalize the inventory of RANKL-regulated genes.

Only one TNF-related gene was regulated by more than tenfold, TNF superfamily member 7 ligand (CD70). CD70 is a type three transmembrane spanning protein that is homologous to TNF$\alpha$, TNF$\beta$, and CD40 [Goodwin et al., 1993]. The CD70 receptor, CD27, was upregulated 2.7-fold by RANKL (data not shown). As CD70 and CD27 are transmembrane proteins and are cognate ligands, they are similar in biochemistry to the RANK–RANKL system. Cell to cell contact is required for CD27 to bind CD70. Expression of both CD70 and CD27 are known in T- and B-cells [Goodwin et al., 1993; Prasad et al., 1997] but have not been observed previously in osteoclasts or osteoclast-like cells treated with RANKL. This suggests that CD70 and CD27 may play a role in differentiation or functional events downstream of the RANKL signal in osteoclast differentiation.

Table III shows a collection of other genes regulated by RANKL within the experiment. The regulated genes vary in nature with few being previously linked to bone, except thrombospondin 2, where knockout mice possessed higher bone mineral density and cortical bone thickness [Kyriakides et al., 1998] and calmodulin 1, where calmodulin inhibitors have been observed to block osteoclast formation in RAW264.7 cells and ovariectomized mice [Zhang et al., 2003]. Regulation of calmodulin 1 (27-fold) after 3-week treatment with RANKL provides further evidence of calcineurin/$Ca^{2+}$-dependant NFATc1 activation in human osteoclastogenesis.

In summary, we have identified a number of genes regulated by RANKL treatment that are potentially important to the maturation and function of osteoclasts. We examined transcription factors in some detail, suggestive of early and late categories, where FBP and c-Jun are early and NFATc1, GABP and NF90 are later in induction profile. In particular, NFATc1 was amenable to repression through cyclosporin A blockade, having a profound effect on calcitonin receptor but not CTSK and TRAP, consistent with NFATc1 having a prime role in later events. In contrast to the inhibition of osteoclast differentiation by cyclosporin A, GM-CSF inhibited the expression of TRAP and CTSK, demonstrating that the two agents, GM-CSF and cyclosporin A, block osteoclastogenesis at different stages.

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