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# Molecular Profiling of Giant Cell Tumor of Bone and the Osteoclastic Localization of Ligand for Receptor Activator of Nuclear Factor κB

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**Giant cell tumor of bone (GCT) is a generally benign, osteolytic neoplasm comprising stromal cells and osteoclast-like giant cells. The osteoclastic cells, which cause bony destruction, are thought to be recruited from normal monocytic pre-osteoclasts by stromal cell expression of the ligand for receptor activator of nuclear factor κB (RANKL). This model forms the foundation for clinical trials in GCTs of novel cancer therapeutics targeting RANKL. Using expression profiling, we identified both osteoblast and osteoclast signatures within GCTs, including key regulators of osteoclast differentiation and function such as RANKL, a C-type lectin, osteoprotegerin, and the wnt inhibitor SFRP4. After *ex vivo* generation of stromal- and osteoclast-enriched cultures, we unexpectedly found that RANKL mRNA and protein were more highly expressed in osteoclasts than in stromal cells, as determined by expression profiling, flow cytometry, immunohistochemistry, and reverse transcriptase-polymerase chain reaction. The expression patterns of molecules implicated in signaling between stromal cells and monocytic osteoclast precursors were analyzed in both primary and fractionated GCTs. Finally, using array-based comparative genomic hybridization, neither GCTs nor the derived stromal cells demonstrated significant genomic gains or losses. These data raise questions regarding the role of RANKL in GCTs that may be relevant to the development of molecularly targeted therapeutics for this disease.** ***(Am J Pathol 2005, 167:117–128)***

Giant cell tumors of bone (GCT) are rare, usually benign connective tissue neoplasms characterized by localized bone destruction. They comprise osteoclast-like giant cells, stromal cells, and CD68-positive monocytes.[1,2] The stromal cells are thought to be the neoplastic component of the tumor, as they can be propagated in culture and stain positive for the proliferation marker, Ki67.[2] There is evidence that the stromal cells are of the osteoblastic lineage[2–4] and are thought to support the recruitment and formation of mature osteoclasts from precursor cells.[5] It is the bone resorptive activity of osteoclasts that causes the destructive osteolysis and consequent morbidity seen in GCT.[6] The molecules that mediate this interaction therefore represent potentially important therapeutic targets.

Osteoclast differentiation and activation is critically dependent on the tumor necrosis factor (TNF) receptor/TNF-like proteins, osteoprotegerin (OPG), and ligand for receptor activator of nuclear factor κB (RANKL).[5,7,8] RANKL expression has been observed in GCT-derived stromal cells,[5] and these cells support osteoclast formation in the RAW264.7 murine monocytic cell line.[9] These and other observations have led to the widely accepted proposition that the expression of RANKL by the neoplastic stromal cell causes the recruitment of osteoclasts.[5] Unexpectedly, the expression of RANKL mRNA and

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**Table 1.** Tumor Type and Numbers of Each Arrayed

| Tumor type | No. arrayed |
|---|---|
| Malignant fibrous histiocytoma | 11 |
| Liposarcoma | 15 |
| Leiomyosarcoma | 9 |
| Synovial cell sarcoma | 4 |
| Giant cell tumor of bone | 9 |

protein has been reported in osteoclasts, including GCTs.[10,11] Perhaps because of methodologic issues affecting immunohistochemical and *in situ* hybridization techniques, these incidental findings have not been widely appreciated, and their significance remains unclear. Because several cell types are involved in the genesis of GCT and a network of 60 proteins are involved in osteoclastogenesis,[6] elucidating the nature and sources of these molecules is important.

To address this issue, we have undertaken genome-wide transcriptional profiling of GCTs. A number of studies have demonstrated the value of molecular profiling of connective tissue neoplasms in general, shedding light on the molecular identity of subclasses of this heterogeneous group of disorders.[12–14] Our studies have focused on human GCT within the context of 48 sarcomas. GCTs form a distinct group within soft-tissue sarcomas by unsupervised hierarchical clustering. After supervised clustering, we identified genes reported to be expressed in osteoclasts and osteoblasts. Identification of the molecules that modulate osteoclastogenesis using genetic profiling and functional assays may lead to the discovery of novel therapeutic targets. As expected, RANKL expression was high in GCTs, and its inhibitor (osteoprotegerin) showed low expression. Using *ex vivo* cellular fractionation methods, we generated osteoclast- and stromal cell-enriched fractions and unexpectedly found that RANKL expression was greater in the osteoclast-enriched hemopoietic fraction. This finding was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR), flow cytometry, and immunohistochemistry, suggesting that the neoplastic component is derived from cells of the osteoclast lineage or that a molecule other than RANKL is responsible for osteoclast recruitment by the stromal cells or that both cell components are derived from a common precursor.

## Materials and Methods

### Processing of Tumor Samples

Forty-eight primary tumor samples were obtained from the Tissue Bank at The Peter MacCallum Cancer Center and St Vincent's Hospital in Melbourne, Australia and the Princess Alexandra Hospital, Brisbane, Australia (Table 1). Separate Institutional Review Board approval was obtained for the collection of samples. The samples are stored at the tissue bank at The Peter MacCallum Cancer Center. Resected specimens underwent blinded pathological review by a dedicated sarcoma pathologist (JS) to ensure at least 80% viable tumor content. The samples obtained and arrayed are shown in Table 1.

### Preparation of Total RNA from Tumor Tissue

Tumor samples were added to Trizol reagent (1 ml per 25 mg of tissue) and homogenized for 30 seconds. The sample was then extracted in Trizol reagent, purified using the Qiagent RNeasy kit (Qiagen Ltd, Sussex, UK), and quantified by spectrophotometry.

### cDNA Synthesis, Transcription, RNA Purification, and Concentration

First-strand synthesis of cDNA was achieved using the Eberwine method.[15] Briefly, total RNA (3 $\mu$g) was reverse transcribed to cDNA using a T7 promoter tagged anchored PolyT primer. Subsequently, second-strand synthesis was achieved using DNA polymerase I (40 U; Promega, Madison, WI), DNA ligase (10 U; Invitrogen, Carlsbad, CA), RNase H (2 U; Invitrogen), dNTP (10 mmol/L each) and 10× DNA polymerase I buffer with an incubation at 16°C for 2 hours. The double-stranded cDNA sample was then used as a template in an *in vitro* transcription reaction using a T7 Megascript kit (Ambion, Austin, TX) following the protocol supplied and then purified using an RNeasy column (Qiagen, Doncaster, Victoria, Australia). The sample was dry eluted to 12 $\mu$l using a vacuum centrifuge and quantified using a spectrophotometer.

### Probe Preparation and Hybridization

Amplified RNA (4–10 $\mu$g) was indirectly labeled by incorporation of amino allyl dUTP (Sigma, Sydney, Australia) during reverse transcription. The sample was then purified using the Qiagen QIAquick Purification kit as per the protocol provided with the exception that the sample is not eluted but left on the column for the subsequent coupling step.

Cyanine-5 fluorophor (Amersham, Castle Hill, NSW, Australia) was resuspended in 20 $\mu$l of 0.1 mol/L Na bicarbonate buffer and added to the center of the column above for all of the test samples. Cyanine-3 fluorophor was used for a reference sample, which consists of pooled and amplified RNA from 11 human tumor cell lines.[16,17] A 1-hour incubation of the column (in the dark) was then required. The sample was then eluted with 80 $\mu$l of water and 13,000 rpm for 1 minute. Five volumes of buffer PB (Qiagen QIAquick kit) was added to each sample.

The eluted cyanine-3-coupled reference sample was added to a new Qiagen column, the eluate was discarded, and then the cyanine-5-coupled test sample was applied to the same column. It was washed with buffer PE, and the sample was eluted with buffer EB (Qiagen). A hybridization mix consisting of tRNA, cot-1 DNA, PolydA, and 50× Denharts containing herring sperm DNA was added to the eluted sample, and the sample was then

concentrated in a centrifugal evaporator. Standard saline citrate (SSC; 20×) and 100% deionized formamide were added to the sample; it was denatured at 100°C for 3 minutes. Sodium dodecyl sulfate (10%) was then added, and the samples were ready to apply to the slide and hybridize for 14 to 16 hours in a humidified chamber at 42°C. The slides were then placed in 0.5× SSC and 0.01% sodium dodecyl sulfate until the coverslips came off and then incubated a further 1 minute in the same solution. The next wash was in 0.5% SSC for 3 minutes, and the final wash was in 0.06% SSC for 3 minutes. The slides were then centrifuged dry at 800 rpm for 5 minutes.

### *Microarray Slides and Analysis*

All experiments were performed using two-color competitive hybridization of fluorescently labeled cDNA to a glass slide array. The cDNA microarrays used contain 9386 cDNA clones representing almost all named genes and several thousand additional expressed sequence tag clones (Unigene build no. 172). Each test sample was compared with a reference sample consisting of pooled RNA from 11 cell lines. The slides were scanned using an Agilent scanner and analyzed using Genepix Pro 4.1 and Genespring software (Silicon Genetics). The following transformations were applied to the raw data. First, adjustments were made for three dye-swapped GCT samples followed by locally weighted regression scatterplot smoothing normalization, and finally, all data were median normalized for gene expression. Spots flagged as absent in more than 3 of 48 samples were excluded from further analysis, yielding 6822 genes (supplemental data at http://ajp.amjpathol.org), and then these genes were filtered on the basis of raw signal (>300), yielding 3376 genes. For supervised hierarchical clustering, we applied Welch's analysis of variance with a *P* value cut-off of 0.05 and Bonferroni's multiple test correction to generate a list of 171 genes that reliably discriminated between histological subtypes (supplemental data at http://ajp.amjpathol.org). For experiments using osteoblast- and osteoclast-enriched cultures, filtered data were manually analyzed using Microsoft Excel (supplemental data at http://ajp.amjpathol.org).

### *Array Comparative Genomic Hybridization*

DNA was extracted from tumor samples or cell culture using standard protocols.[18] A total of 3 μg of DNA was sent to the Microarray Shared Resource at the Comprehensive Cancer Center, University of California, San Francisco, and subjected to array-based comparative genomic hybridization as previously described.[19]

### *Subfractionation of GCT into Giant Cell and Stromal Fractions*

Selected GCT samples were subfractionated according to the method published by Atkins et al.[5] Briefly, GCT samples were used fresh, treated with collagenase, and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cultured sample consisted of giant cells in a stromal cell mass. The stromal cell fraction was removed by trypsin digestion (0.1% w/v in phosphate-buffered saline (PBS)) and replated into a separate flask while the giant cells and mononuclear fan-shaped cells remained attached to the flask. After 48 hours, the process was repeated until giant cells were no longer seen in the stromal fraction, yielding a stromal cell-enriched fraction and a giant cell-enriched fraction.

For bioassays of RANKL production, we used RAW264.7 cells, a monocytic cell line that forms osteoclasts in the presence of recombinant human RANKL.[20] Briefly, either supernatants from primary stromal cell cultures were added to RAW264.7 cells or stromal cells were co-cultured with RAW264.7 cells. RAW264.7 cells were then cultured for up to 10 days before staining for tartrate-resistant acid phosphatase (TRAP) as described previously.[20] Control cultures were conducted using recombinant human RANKL to ensure that these cells formed TRAP-positive osteoclast like cells.

### *PCR Samples and Conditions*

GCT, osteoblast, and osteoclast cDNAs were diluted 1 in 25, and 1 μL was used for PCR. cDNA was amplified using the PCR primers listed in Table 2 to generate mRNA products encoding the specific human genes. The 25-μL PCR reaction consisted of 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT), 0.4 mmol/L dNTPs (Pharmacia Biotech, Uppsala, Sweden), 1.5 mmol/L magnesium chloride, 50 nmol/L of each primer, and 1× PCR buffer and was made to volume with RNase-free water. The conditions used were: 95°C for 5 minutes to activate the polymerase and 40 cycles of 95°C for 30 seconds to denature, 50 to 56°C gradient for 30 seconds to anneal, and 72°C for 30 seconds for extension; the 40 cycles were followed by an additional extension of 72°C for 5 minutes, and then the samples were kept at 4°C before the addition of loading buffer (6×). They were then run on a 1.8% agarose gel and visualized via ethidium bromide staining.

### *PCR Primers*

Some of the primer sequences used were obtained from Atkins et al,[5] whereas the remainder were generated from sequences corresponding to accession numbers for cDNAs contained in our microarrays.

### *Flow Phenotyping*

Fresh GCT samples were collagenase treated as described above. Cells were disaggregated and passed through a 19-gauge needle to remove debris. All subsequent steps were carried out at 4°C. Cells were incubated with PerCP-conjugated monoclonal anti-human CD45 (DAKO) for 15 minutes, followed by washing (PBS and 1% bovine serum albumin). After fixation in 2% paraformaldehyde for 10 minutes on ice, cells were incubated with monoclonal anti-human RANKL (MAB626; R&D Systems) at 1 μg/ml for 10

**Table 2.** Primers to Osteoclast, Osteoblast, and Giant Cell Tumor Markers Selected through Microarray Expression Signatures as well as Published Data[5]

| Target gene | Sense (5′ to 3′) | Antisense (5′ to 3′) | Melting temperature (°C) | Product (bp) |
|---|---|---|---|---|
| OPG | TGCTGTTCCTACAAAGTTTACG | CTTTGAGTGCTTTAGTGCGTG | 62 | 435 |
| M-CSF | CAGTTGTCAAGGACAGCAC | GCTGGAGGATCCCTTCGGACTG | 58 | 670 |
| SFRP4 | TGCTGCCGACTGGAGTTTG | TGAGGTCCCACGTTTACCC | 63 | 529 |
| RANKL | AATAGAATATCAGAAGATGGCACTC | TAAGGAGGGGTTGGAGACCTCG | 62 | 668 |
| C-type lectin | TCCAGGCTGTCTCTTCCACG | TGTGCCTATCTGGTGCCTCTG | 65 | 557 |
| CTR | GCAATGCTTTCACTCCTGAGAAA | AGTGCATCACGTAATCATATATC | 58 | 782 |
| TRAP | CTGGCTGATGGTGCCACCCCTG | CTCTCAGGCTGCAGGCTGAGG | 65 | 469 |
| CSF1R | GCTTGGCATGGTCAGGGAAT | GGGCCCTGGGATGACTTTCT | 65 | 898 |
| Cathepsin K | GATCACTGGAGCTGACTTCCG | GGGCTCTACCTTCCCATTCTG | 66 | 470 |
| OSF1 | TCCTAGGAGGCGACGGTTGT | CGTGGCAAGCCCAGTATAAGG | 63 | 495 |
| OSF2 | GGACCAAGGCCCAAATGTCT | CCCATGGATGATTCGAGCA | 63 | 653 |
| Osteonectin | GCAAGAAGCCCTGCCTGAT | GGGAATTCGGTCAGCTCAGA | 62 | 548 |
| GAPDH | CACTGACACCTTGGCAGTGG | CATGGAGAAGGCTGGGGCTC | 60 | 414 |

minutes. After washing, cells were blocked in PBS containing 10% mouse serum for 10 minutes on ice. After three more washes, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse immunoglobulin (Jackson Immunoresearch) for 10 minutes before three washes in PBS/bovine serum albumin. Appropriate negative controls were included. Cells were then subject to flow cytometry (FACScalibur, Becton Dickinson).

### *Immunohistochemistry*

Paraffin-embedded tissue sections were cut and mounted onto slides. These were stained with TRAP using an alkaline leukocyte phosphatase kit (Sigma), CD68 antibody, CD45 antibody, CD4 antibody, RANKL antibody, and vimentin. Immunostaining for RANKL was performed using the same antibody described above (MAB626).

## *Results and Discussion*

### *Expression Profiling of Giant Cell Tumor of Bone*

We undertook expression profiling of 48 sarcomas, including 9 GCTs. Using unsupervised hierarchical clustering, GCTs formed a distinct subgroup, consistent with the striking morphology of these tumors (Figure 1A). Supervised clustering (Figure 1B), based on genes that accurately discriminated between subclasses of sarcomas ($P < 0.01$), reinforced the impression that GCTs formed a remarkably homogeneous subgroup. A full discussion of the remaining classes of sarcoma lies beyond the scope of this paper, but the raw data are provided as supplemental information (http://ajp.amjpathol.org). We next examined those genes most highly expressed in GCTs, ranked according to their median expression (Table 3). This gene list comprises the top 20 genes and their median expression in GCTs. As expected, the list included several genes known to be highly expressed in osteoclasts, including cathepsin K[21], the brain isoform of creatine kinase,[22] calcineurin-dependent nuclear factor of activated T-cells,[23] and subunit of a membrane-associated ATP-dependent proton pump.[24] Additionally, a number of osteoblast-related genes were identified, including decorin, lumican, and collagen IX, consistent with the proposition that the stromal element in GCTs is of osteoblastic origin. Almost all remaining genes identified in our study have been implicated in osteoclast biology. Foremost among these, RANKL (tumor necrosis factor superfamily member 11) was extremely highly expressed in these tumors, whereas OPG was expressed at very low levels (data not shown). Because OPG is a decoy inhibitor of RANKL signaling, this is consistent with a potent osteoclastogenic signal in GCTs. C-type lectin (member 6) belongs to a class of molecules previously identified as inhibitors of osteoclast formation (osteoclast-inhibitory lectin).[25] C-type lectin 6 and murine osteoclast-inhibitory lectin are 23% identical and 51% similar at the protein level, suggesting overlapping functions. Similarly, SFRP4 belongs to a family of secreted frizzled-related proteins, one of which (SFRP1) has aroused interest as an inhibitor of osteoclastogenesis.[26,27] It is not clear whether C-type lectin 6 or SFRP4 are acting as inhibitors or activators of osteoclast formation in GCTs, because homologs may act in an antagonistic function, as seen with OPG and RANKL. Decysin 1 is a member of the ADAM family (a disintegrin and metalloprotease), which has been implicated in regulation of osteoclast recruitment and function.[28] Protein kinase A mediates signals downstream of a number of osteoclastogenic stimuli, including parathyroid hormone and prostaglandins.[29,30] Lymphotoxin-$\beta$ receptor binds and transduces signals from tumor necrosis factor family proteins, to which RANKL and OPG belong.[31,32] Thrombospondin-1 has been reported to stimulate bone resorption by osteoclasts *in vitro*[33], whereas there have been two studies indicating that TYRO3 and its ligand, GAS6, stimulate osteoclast bone resorption.[34,35] Our studies provide strong evidence that these molecules, which are expressed at high levels in GCTs, play key roles in osteoclast recruitment and function in human disease.

### *Molecular Profiling of Stromal- and Osteoclast-Enriched Fractions of GCT*

We sought to confirm the stromal origin of RANKL in GCTs by fractionating the stromal and hemopoietic com-

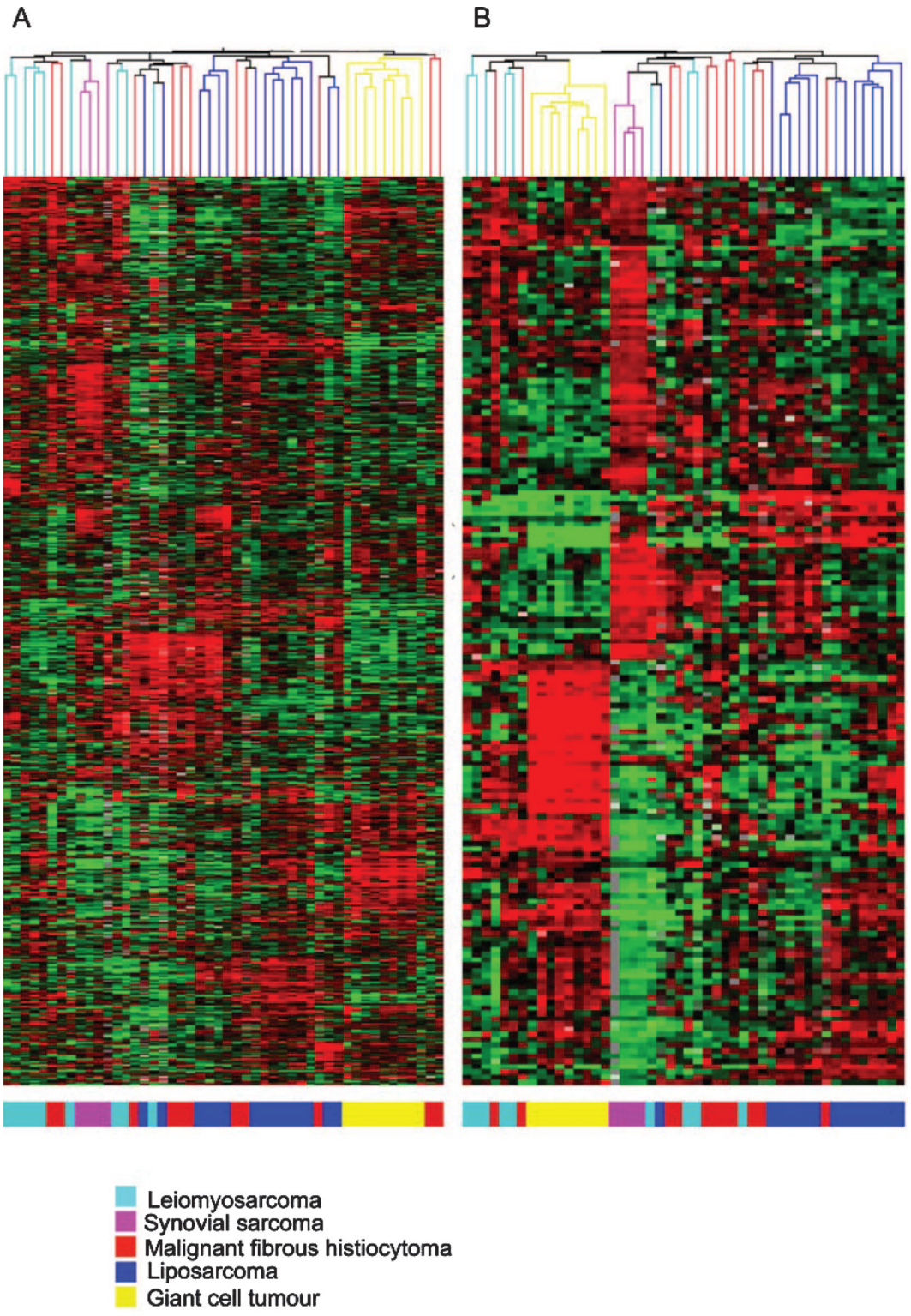


**Figure 1.** Gene expression heatmaps for leiomyosarcoma, synovial sarcoma, malignant fibrous histiocytoma, liposarcoma, and giant cell tumor using both unsupervised (**A**) and supervised (**B**) analysis.

ponents, followed by expression profiling of the stromal-enriched and hemopoietic-enriched cultures.[5] This crude fractionation procedure resulted in isolation of relatively pure populations of stromal cells, but osteoclast-enriched populations tended to demonstrate residual stromal cell contamination, probably reflecting the nature of the purification process (Figure 2, A and B). Gene expression signatures were obtained that reflect relative enrichment in osteoclast and osteoblast-like cellular components of GCT (Figure 3 and Table 4). Specifically, we observed

**Table 3.** Top 24 Genes Most Highly Expressed in Giant Cell Tumor of Bone by Microarray Analysis

| Class | Gene name | Accession no. | Median gene expression |
|---|---|---|---|
| Stromal phenotype genes | Matrix metalloproteinase 13 (collagenase 3) | N69322 | 63 |
| | Decorin | H64138 | 56 |
| | Collagen type XI $\alpha$1 | R52907 | 51 |
| | Collagen, type I, $\alpha$2 | AA490172 | 18 |
| | Lumican | AA447781 | 32 |
| | Collagen, type V, $\alpha$1 | R75635 | 21 |
| Osteoclast phenotype genes | Cathepsin K (pycnodysostosis) | R00859 | 74 |
| | Acid phosphatase 5, tartrate resistant | R08817 | 36 |
| | Creatine kinase, brain | AA894557 | 34 |
| | Thymidine kinase 1, soluble | AA778098 | 18 |
| | Nuclear factor of activated T-cells, c1 | AA679278 | 24 |
| | Colony stimulating factor 1 receptor | AA284954 | 23 |
| | ATPase, H+ transporting, lysosomal V0 protein a isoform 3 | AI359884 | 19 |
| Osteoclast regulatory genes | Thrombospondin 2 precursor | H38240 | 50 |
| | TNF superfamily, member 11 (RANKL) | AA504211 | 42 |
| | Secreted frizzled-related protein 4 | AA487193 | 41 |
| | C-type lectin, superfamily member 6 | AA677149 | 32 |
| Unknown role | *Homo sapiens* cDNA clone IMAGE:122159 3′, mRNA sequence | T98612 | 38 |
| | Hypothetical protein, estradiol-induced | R36989 | 26 |
| | TYRO protein tyrosine kinase binding protein | H12338 | 24 |
| | *H. sapiens* cDNA clone IMAGE:51447 3′, mRNA sequence | H20822 | 23 |
| | lymphotoxin-$\beta$ receptor (TNFR superfamily, member 3) | AA454646 | 20 |
| | Vanin 1 | AA983530 | 19 |
| | Coagulation factor XIII, A1 polypeptide | AA449742 | 18 |

relative enrichment in the osteoclastic fraction of markers known to be highly expressed in monocytes and osteoclasts (cathepsin K, CD4, CD68, ATP-dependent proton pump subunits, and $\alpha$V integrin) and relative depletion in stromal or osteoblastic genes (decorin, collagens, and lumican). Notable among genes enriched in the osteoclast fraction is the microphthalmia transcription factor (Mitf) family member, TFEC. Mice deficient in *mitf,* the canonical member of the Mitf family, closely resemble mice lacking cathepsin K and have dysfunctional osteoclasts leading to osteopetrosis.[36] Although *Tfec*-deficient mice appear phenotypically normal, Mitf, TFEB, TFEC, and TFE3 are functionally redundant basic helix-loop-helix-leucine zipper transcription factors that form homo- and heterodimeric complexes on DNA and have been shown to regulate expression of cathepsin K.[37] Our data suggest that TFEC plays an important role in human osteoclast biology. Another highly enriched protein in the osteoclast-enriched fraction is GRB2-binding protein, previously reported to be abundant in osteoclasts,[38] and cystatin B, which is found in osteoclasts and may regulate cathepsin activity.[39] The stromal fraction expressed osteoblastic or mesenchymal cell markers, including fibroblast growth factor receptor 1, osteoglycin, lumican, collagen XI, and decorin.

## *Expression of RANKL Is Enriched in the Osteoclast Fraction*

Unexpectedly, we observed striking segregation of signaling molecules with known or potential roles in osteoclast differentiation and function in the osteoclast-enriched fraction, including RANKL, SFRP4, and C-type lectin 6 (Figure 3 and Table 4). In contrast, we observed slight relative enrichment of the osteoblastic fraction for the inhibitor of osteoclastogenesis, OPG. To confirm this result, we undertook semiquantitative RT-PCR for osteoblast and osteoclast markers, as well as regulators of

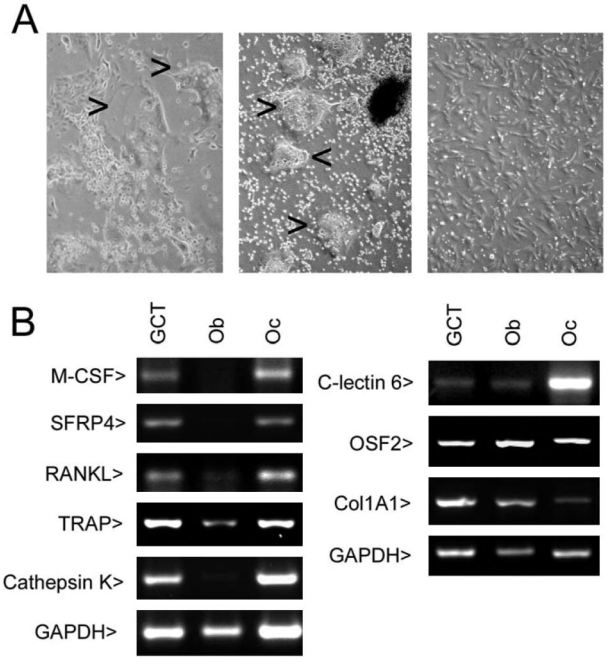


**Figure 2.** Cell fractionation studies. **A:** Light microscopy showing typical cultures of primary disaggregated giant cell tumor (GCT; **left panel**), osteoclast-enriched cultures (**middle panel**), and stromal cell cultures (**right panel**). **Arrows** indicate giant cells. **B:** RT-PCR for expression of regulatory genes, osteoclast and osteoblast markers in primary giant cell tumor cultures, and osteoblast- and osteoclast-enriched fractions of GCT. This experiment was performed three times with similar results using independent samples to those used in Table 4.

| OC-enriched genes | Stromal cell enriched genes |
|---|---|
| transcription factor EC | melanoma antigen, family A, 4 |
| colony stimulating factor 1 receptor | transcriptional co-repressor of retinoid and thyroid hormone action (SMRT) |
| S100 calcium binding protein A3 | Collagen type XI apha 1 |
| GRB2-associated binding protein 1 | collagen, type I, alpha 2 |
| KIAA1199 protein | extracellular matrix protein 2, female organ and adipocyte specific |
| solute carrier family 22 (organic cation transporter), member 1 | decorin |
| nuclear factor of activated T-cells, calcineurin-dependent 1 | endothelial differentiation, sphingolipid G-protein-coupled receptor, 1 |
| regulator of G-protein signalling 1 | hypothetical protein, estradiol-induced |
| tumor necrosis factor (ligand) superfamily, member 11 | H factor (complement)-like 1 |
| C-type lectin, superfamily member 6 | lumican |
| ribonuclease, RNase A family, k6 | phospholipase A2 receptor 1, 180kDa |
| selectin P ligand | BASIC FIBROBLAST GROWTH FACTOR RECEPTOR 1 PRECURSOR |
| chemokine (C-C motif) receptor 1 | complement component 1, r subcomponent |
| CD4 antigen (p55) | TSHUP2 thrombospondin 2 precursor - human |
| adenylate kinase 5 | KIAA0471 gene product |
| A kinase (PRKA) anchor protein 3 | ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin) |
| regulator of G-protein signalling 10 | PDZ domain containing 3 |
| CYSTATIN B (HUMAN);, mRNA sequence. | epithelial membrane protein 1 |
| ATPase, H+ transporting, lysosomal V0 protein a isoform 3 | fibroblast activation protein, alpha |
| potassium voltage-gated channel, subfamily F, member 1 | coagulation factor XIII, A1 polypeptide |
| lymphocyte-specific G protein-coupled receptor) | osteoglycin (osteoinductive factor, mimecan) |
| creatine kinase, brain | MASP-2 PROTEIN. ;, mRNA sequence. |
| ubiquitin fusion degradation 1-like | integrin, beta-like 1 (with EGF-like repeat domains) |
| hypothetical protein DKFZp434L142 | |
| potassium channel, subfamily K, member 7 | |
| CDC20 cell division cycle 20 homolog (S. cerevisiae) | |
| cathepsin K (pycnodysostosis) | |
| secreted frizzled-related protein 4 | |
| dermatan sulfate proteoglycan 3 | |
| Homo sapiens cDNA FLJ10130 fis, clone HEMBA1003035. | |
| chondroadherin | |
| ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 2 | |
| CYSTATIN B (HUMAN);, mRNA sequence. | |

**Figure 3.** Tabular representation of genes most highly expressed in osteoclast- and stromal cell-enriched fractions of giant cell tumor of bone. Genes highlighted in red are genes reported to be expressed in osteoclasts; in blue, expressed in stromal cells; in green, genes implicated in signaling of osteoclast formation or function (see text for details).

osteoclast formation. As noted above, these data suggest that we have obtained relatively pure populations of stromal cells, whereas the osteoclast-enriched populations contained significant numbers of residual stromal cells (Figure 2, A and B). Consistent with the expression profiling data, the expression of RANKL, SFRP4, and C-type lectin 6 again segregated with markers of the osteoclast phenotype, and these molecules were almost undetectable in the stromal cell-enriched fraction (Figure 2B). This experiment has been repeated three times with similar results. We confirmed these semiquantitative PCR results using real-time quantitative PCR on an independent set of cultures, demonstrating the highest levels of expression of RANKL in unfractionated GCT (relative expression, 3.6, normalized to cystatin A), with intermediate signal in the osteoclast fraction (0.58) and the lowest in the osteoblast fraction (0.08).

As a final assay for RANKL as a secreted or membrane-bound signal mediating the recruitment of monocytic cells by stromal cells, we used RAW264.7 cells as a bioassay for RANKL production. RAW264.7 cells form TRAP-positive mono- and multinucleated osteoclast-like cells in the presence of recombinant human RANKL (in our hands, between 10 and 50 TRAP-positive cells/high-power field[20]). However, in repeated experiments, neither GCT stromal cell supernatants nor co-culture with GCT-derived stromal cells resulted in the formation of TRAP-positive cells (data not shown). These data support the view that primary cultures of GCT-derived stromal cells do not secrete or express at the cell surface biologically significant levels of RANKL.

## In Situ *RANKL Expression in GCTs*

Given the presence of contaminating stromal cells in the osteoclast-enriched fraction, we could not exclude the possibility that RANKL expression occurred in stromal cells, but only in the presence of osteoclast-like cells. To test this possibility, we undertook two experiments examining RANKL expression *in situ*. First, we undertook a flow cytometric study of disaggregated GCTs, specifically examining co-expression of CD45 (a pan-hemopoietic marker) and RANKL. We found that CD45 was expressed on osteoclasts in GCTs, along with high levels of CD68 and low levels of CD4, consistent with the hemopoietic origin of these cells. We found that approximately 50% of CD45-positive cells expressed RANKL, whereas we were unable to detect significant expression of RANKL on CD45-negative cells (Figure 4). Second, we immunostained GCTs for RANKL, CD4, CD45, and CD68. These markers were chosen because CD4 and CD68 were observed to be expressed in the osteoclast fraction (Table 4; data not shown), and CD45 is a pan-leukocyte marker consistent with the hemopoietic origin of osteoclasts. Giant cells stained for CD4 and CD45 at the plasma membrane and for CD68 in the cytoplasm (Figure 5), as well as tartrate-resistant acid phosphatase (data not shown). By contrast, these markers were absent from stromal cells within these tumors, which stained strongly with vimentin (data not shown). RANKL immunoreactivity was noted most strongly surrounding osteoblasts adjacent to newly forming bone at the periphery of the GCT (Figure 6, A and B) and in giant cells in these studies

**Table 4.** Genes Encoding Cytokines or Receptors Found Overexpressed (Greater Than 2-fold Enriched) in Either Primary GCTs or Osteoblast-Enriched or Osteoclast-Enriched Cell Populations

| | Accession No. | Median expression in osteoblast-enriched population | Median expression in osteoclast-enriched population | Relative median expression | Unigene name |
|---|---|---|---|---|---|
| Giant cell tumor | AA036881 | | | 5.56 | Chemokine (C-C motif) receptor 1 |
| | AA437226 | | | 2.63 | Interleukin 10 receptor, $\alpha$ |
| | N54821 | | | 2.51 | Interleukin 2 receptor, $\gamma$ |
| | AA449440 | | | 2.37 | Interferon-$\gamma$ receptor 2 |
| Osteoclast-enriched | AA504211 | 3.42 | 45.06 | 13.18 | TNF member 11 (RANKL) |
| | AA150507 | 0.96 | 7.56 | 7.88 | Interleukin 1, $\beta$ |
| | AA779457 | 1.11 | 8.12 | 7.32 | Bone morphogenetic protein 5 |
| | AI569017 | 1.24 | 7.55 | 6.08 | Bone morphogenetic protein 2 |
| | AW073000 | 1.71 | 9.33 | 5.45 | TNF receptor member 14 |
| | N54821 | 0.43 | 1.74 | 4.08 | Interleukin 2 receptor, $\gamma$ |
| | AA936768 | 1.22 | 3.77 | 3.10 | Interleukin 1, $\alpha$ |
| Osteoblast-enriched | AA463225 | 0.42 | 0.10 | 4.06 | Bone morphogenetic protein 4 |
| | AA194983 | 0.44 | 0.14 | 3.20 | TNF receptor member 11b (OPG) |
| | AA253464 | 0.45 | 0.16 | 2.41 | Dickkopf 1 |
| | W73473 | 0.42 | 0.18 | 2.39 | Bone morphogenetic protein 7 |
| | AA490494 | 1.11 | 0.47 | 2.34 | TNF receptor member 21 |
| | AA464525 | 6.14 | 2.70 | 2.27 | Interleukin 1 receptor, type I |
| | AA456160 | 1.10 | 0.50 | 2.19 | Fibroblast growth factor receptor 2 |
| | W15390 | 1.38 | 0.63 | 2.18 | Bone morphogenetic protein receptor IA |

Note that relative median expression indicates expression relative to remaining sarcoma types for primary GCT and expression relative to noncognate population for osteoblast- and osteoclast-enriched populations.

(Figure 6, C to F). There was little RANKL staining in the mononuclear population in the bulk of the GCT. Interestingly, the pattern of RANKL staining was not membrane associated (for comparison, see CD45 and CD4 staining in Figure 5, B and C). Taken together, these data suggest that RANKL mRNA and protein are expressed by osteoclasts within GCTs. RANKL is also detectable in some stromal cells, although it appears that levels are depleted in isolated stromal cell populations. The progressive loss of expression of RANKL and osteoblast markers by the isolated stromal fraction raises the possibility that there may be reciprocal interactions between the stromal cell and the osteoclast that support not only osteoclast formation, but maintenance of the osteoblast phenotype. The highest levels of RANKL expression appeared to arise at the margins of the tumor where new bone formation was most evident.

## *Other Molecules Involved in Stromal-Hemopoietic Cell Signaling*

In addition to those described above, several molecules have been implicated in signaling between stromal cells and osteoclast progenitors (reviewed by Boyle et al[40]). Pro-resorptive factors include monocyte colony stimulating factor, interleukins-1, -6, and -11, tumor necrosis factor-$\alpha$, 1,25(OH)2 vitamin D3, parathyroid hormone, and parathyroid hormone-like protein, prolactin, corticosteroids, oncostatin M, and leukemia inhibitory factor. Anti-resorptive factors include platelet-derived growth factor, bone morphogenetic proteins 2 and 4, calcitonin, estrogens, thrombopoietin, and transforming growth factor-$\beta$. Molecules involved in regulation of stromal osteoblast differentiation and function include fibroblast growth factors[41] and bone morphogenetic proteins (BMPs).[42] We systematically examined expression in GCTs and in the isolated cell populations of these and other genes (Table 4). In many cases, the genes were not represented on the arrays or were removed during filtering because of low expression. Of the remaining 6822 genes, we observed overexpression of the chemokine receptor 1 (CCR1) in primary GCT. CCR1 is a seven-transmembrane G protein-coupled receptor involved in monocyte and macrophage function. Ligands that bind CCR1, including RANTES, macrophage inhibitory protein-$\alpha$, and monocyte chemotactic protein-3, have been reported to

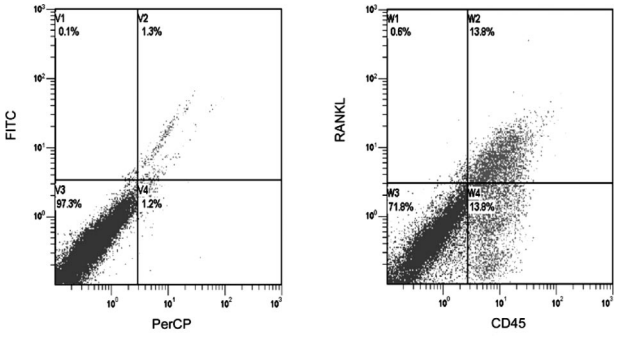


**Figure 4.** Flow phenotyping of GCT with FITC and PerCP (controls) and RANKL and CD45. There is an increase in the cell population staining positive for both RANKL and CD45, and there are no cells staining RANKL positive and CD45 negative compared with the control samples with FITC and PerCP.

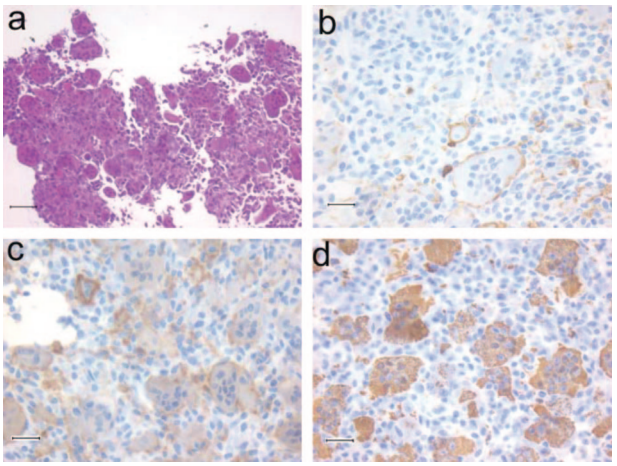


**Figure 5.** Confirmation of expression profiling data by immunohistochemistry of paraffin-embedded fixed giant cell tumor of bone. **a:** Conventional H&E section; **b:** CD4 staining for osteoclasts; **c:** CD45, which can be also used as an osteoclast cell surface marker; and **d:** CD68, which is a hemopoietic cell or osteoclast marker. Bar = 50 $\mu$m in **a**, and 25 $\mu$m in **b** to **d**.

promote osteoclast differentiation and motility.[43] We observed modestly increased expression of receptors for interleukins-2 and -10, and interferon-$\gamma$ (Table 4). Interestingly, we did not observe increased expression of the ligands for these receptors, although in many cases, these were not represented in our dataset.

In the isolated cell cultures, expression of RANKL dominates the list of signaling molecules in osteoclast-enriched cultures, whereas osteoprotegerin expression was relatively enriched in the stromal cell fraction (Table 4). Interestingly, interleukin 1-$\beta$ and -$\alpha$ were relatively overexpressed in the osteoclast-enriched cultures. Interestingly, we also observed relative enrichment for BMP-5 and BMP-2 in osteoclastic populations, whereas the stromal cell-enriched cultures were relatively enriched for the type 1A BMP receptor. This is consistent with reciprocal signaling from the hemopoietic compartment to the stromal compartment. Tian et al[44] postulated that the elaboration of the Wnt signaling pathway inhibitor, dickkopf 1, may maintain osteoblasts in an immature state, in which expression of RANKL is increased.[45] dickkopf 1 is not relatively overexpressed in primary GCT (median expression = 1 relative to other sarcomas) but is relatively enriched in the stromal cell fraction. Other molecules involved in regulating stromal cell differentiation and function expressed at higher levels in stromal cells than the osteoclastic fraction included BMP-4 and -7 and the type 2 receptor for fibroblast growth factor.

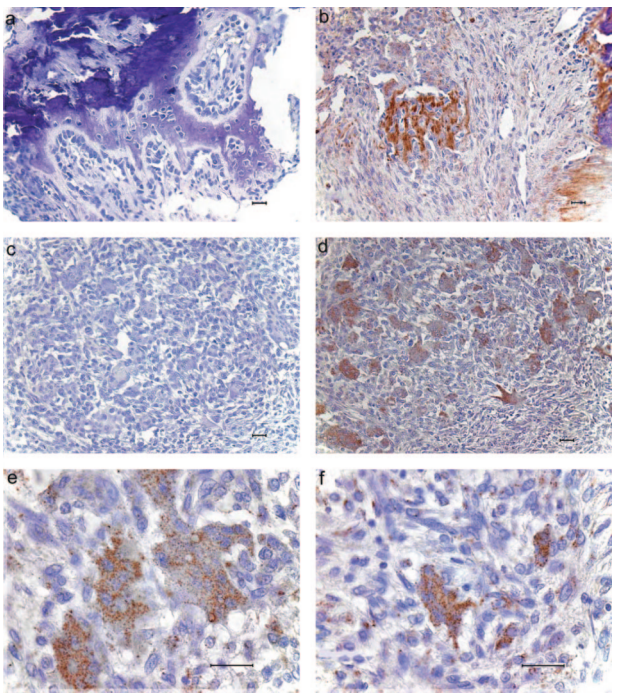


**Figure 6.** RANKL antibody staining of giant cell tumor of bone. Antibody control GCT section (**a**) and RANKL antibody staining in parallel sections (**b**) showing specific staining adjacent to osteoblasts clustering at a site of newly forming bone. Antibody control (**c**) and RANKL staining (**d**) of parallel sections from a tumor showing specific staining of osteoclasts (low magnification). **e** and **f:** Higher magnification of RANKL-stained osteoclasts, showing granular cytoplasmic staining. Scale bar = 25 $\mu$m.

## *Conclusions*

The observation that RANKL mRNA and protein are highly expressed in osteoclasts in GCT of bone is significant, because they are not consistent with the prevailing pathogenetic model of GCTs.[46] This holds that the osteoclast component of GCTs is passively recruited by the production of RANKL by the stromal neoplastic component,[9] based on studies that have shown clearly RANKL expression in GCT stromal cells, and that these cells are capable, when stimulated appropriately, of supporting the formation of osteoclasts.[5] RANKL is necessary and sufficient for the generation of osteoclasts from monocytic precursors, because the genetic deletion of RANKL results in failure of osteoclastogenesis, and RANKL will substitute for the requirement of stromal cells in co-culture models of osteoclastogenesis.[8,47] However, a number of observations are difficult to reconcile with this model. Close inspection of data from several studies confirms both RANKL mRNA and protein expression have been detected in osteoclasts, including multinucleated giant cells in GCTs or the related osteoclast-enriched tumor, pigmented villonodular synovitis,[10,11,48,49] although surprisingly this observation was commented on only in one paper. Moreover, inoculation of GCT-derived stromal cells into SCID mice generated mineralized bone without an excess of osteoclasts or evidence of osteolysis, consistent with an osteoblastic origin, but not inherent osteoclastogenic properties.[4]

How good is the evidence that the stromal component of GCT represents the neoplastic element? First, GCT belongs to a class of benign tumor characterized by a giant cell component, including aneurysmal bone cyst, pigmented villonodular synovitis, and giant cell tumor of tendon sheath.[46] In these tumors, the giant cell element is usually the minor component, and the histological appearance of the tumor is dominated by the stromal element. In many cases, the giant cell component may be difficult to identify. Second, the stromal element is more readily cultured *ex vivo*, although interestingly monocytic

or osteoclast-like cell lines have successfully been derived from GCT.[50,51] These lines are capable of spontaneously generating osteoclast-like cells, suggesting that they autonomously possess all of the molecular signals required for osteoclastogenesis, consistent with the expression of RANKL by the hemopoietic component. We are not aware of stromal cell-derived cell lines that have been generated from GCT. Third, several studies have reported clonal karyotypic abnormalities in the stromal cells derived from GCT. The most frequent genetic event observed has been telomeric fusions, with scattered reports of other abnormalities. Telomeric fusions or associations are thought to represent noncovalent interactions at chromosomal ends, and their significance in cancer is fully understood. A reduction in telomere length (average loss of 500 bp) has been reported in giant cell tumor cells when compared with leukocytes from the same patients.[52] The telomeres commonly affected by fusions include 11p, 13p, 14p, 15p, 19q, 20q, and 21p.[53–57] By contrast, the osteoclast-like cell line, UISO-GCT-1, demonstrated a hypodiploid, hypotetraploid, and multiploid karyotype.[9] There is a lack of good karyotypic data on the osteoclast-like cells derived from GCTs, in large part due to the difficulty in *ex vivo* culture required for generation of metaphase spreads. We have undertaken ploidy studies using flow cytometry of disaggregated GCTs, and we have not observed an aneuploid population (data not shown). To identify smaller scale recurrent gains or losses, we have used array-based comparative genomic hybridization to examine a limited number of GCTs and the derived stromal cell populations. We did not observe any evidence of deletions or amplified regions in either the primary GCT or the stromal populations (data not shown). These data suggest that the neoplastic fraction within GCTs is not characterized by gross disturbances to genomic integrity. Proof that either the stromal or giant cell compartment (or both) of GCT is neoplastic will come from the demonstration of pathognomonic mutations, and it is likely that these will be subtle.

In summary, our observations are inconsistent with the currently accepted model that the simple elaboration of RANKL by neoplastic stromal cells causes the formation of osteoclast-like cells in GCTs.[46] There appear to be three alternative interpretations. First, it is possible that RANKL is a necessary downstream effector of osteoclast formation, induced in both stromal and hemopoietic populations by neoplastic stromal cell expression of an unidentified molecule. This model represents a variation of the existing model, positing that the stromal cell still drives osteoclast formation, but that RANKL expression is part of the mechanism rather than the initiating signal. Second, it remains formally possible (although unlikely) that the neoplastic component of the GCT is derived from the hemopoietic compartment, driving osteoclast formation by autocrine stimulation by RANKL. The stromal component may represent the abortive attempt by reactive stromal osteoblasts to repair the bone destruction caused by the neoplastic hemopoietic component. Finally, an unknown common precursor could give rise to all of the cellular elements of GCTs. This intriguing possibility postulates that the neoplastic precursor possesses the ability to manifest both stromal and osteoclastic phenotypes. There is a precedent in biphasic synovial sarcoma, in which both epithelial and stromal elements are demonstrable.[46] In this model, aberrant expression of RANKL stimulates, in an autocrine/paracrine manner, the stochastic commitment to expression of markers of the osteoclast lineage. Although much work may be required to dissect these alternatives, it is important to determine whether the expression of RANKL is necessary to the formation of osteoclast-like cells, because novel inhibitors of RANKL signaling are being considered as potential therapeutic agents in this otherwise chemo-refractory disease.

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