See related Commentary on page 2248

BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Extremely High Expression of Antisense RNA for Wilms' Tumor 1 in Active Osteoclasts

Suppression of Wilms' Tumor 1 Protein Expression during Osteoclastogenesis

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Wilms' tumor 1 (WT1), a zinc-finger transcription regulator of the early growth response family, identified as the product of a tumor suppressor gene of Wilms' tumors, bears potential ability to induce macrophage differentiation in blood cell differentiation. Herein, we examined the involvement of WT1 in the regulation of osteoclastogenesis. We detected a high level of WT1 protein expression in osteoclast precursors; however, WT1 expression was markedly suppressed during osteoclastogenesis. We examined expression of WT1 transcripts in bone tissue by RNA *in situ* hybridization. We found a high level of antisense transcripts in osteoclasts actively resorbing bone in mandible of newborn rats. Expression of antisense WT1 RNA in mandible was also confirmed by Northern blot analysis and strand-specific RT-PCR. Overexpression of antisense WT1 RNA in RAW-D cells, an osteoclast precursor cell line, resulted in a marked enhancement of osteoclastogenesis, suggesting that antisense WT1 RNA functions to suppress expression of WT1 protein in osteoclastogenesis. High level expression of antisense WT1 RNA may contribute to commitment to osteoclastogenesis, and may allow osteoclasts to maintain or stabilize their differentiation state. (*Am J Pathol* 2016, 186: 2317–2325; <http://dx.doi.org/10.1016/j.ajpath.2016.05.005>)

Human genome projects have shown that a relatively small fraction of genomic sequences are used for encoding proteins. For many years, other genomic sequences that do not encode proteins had been thought to be meaningless DNA.¹ However, discovery of regulatory RNAs has clarified the important roles of these DNAs as templates for regulatory RNAs.^{2–4} miRNAs, regulatory RNAs transcribed from such noncoding genomic DNA, provide an abundant source of small interference RNAs that specifically silence gene expression of specific genes.² Recently, antisense RNAs, which are generally transcribed from the opposite DNA strand to that for mRNAs, have been recognized as important regulatory RNAs.^{5,6} Through several molecular mechanisms involving the transcription collision model, transcription of the antisense RNA results in specific blocking of the

mRNA.⁷ One of the most profoundly investigated antisense RNA is Tsix, which mediates imprinting in X-chromosome inactivation.^{8–12} Antisense RNAs also play important roles in inducing mutation in hypervariable regions of immunoglobulin variable region genes in B-cell development.^{13,14} Continued progress in this area has demonstrated crucial roles of regulatory RNAs in a range of biological processes, including cell growth, differentiation, maintenance of stem cell populations, and tumorigenesis.^{15–17}

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The Wilms' tumor suppressor gene encodes a zinc finger transcription factor belonging to the EGR family, Wilms' tumor 1 (WT1), which is known to be implicated in both normal kidney development and tumorigenesis.¹⁸ In normal kidney development, WT1 is required for the development of nephrons. Recently, it has also been shown that WT1 acts as a critical transcription factor in development of the cardiovascular system.¹⁹ During hematopoiesis, WT1 is highly expressed in hematopoietic progenitors,²⁰ and most human acute leukemia contains high levels of wild-type WT1 transcripts.^{21–23} It has been reported that WT1 acts as a potent inducer of macrophage differentiation.²⁴

Osteoclasts, which share a common progenitor cell with macrophages, are primary bone resorbing cells.²⁵ Transcription factors having the ability to induce macrophages are expected to play regulatory roles in osteoclast differentiation. We speculated that osteoclastogenesis might be regulated by WT1, as this transcription factor has the ability to induce macrophage differentiation.²⁴ Herein, in an examination of the possible involvement of WT1 in control of osteoclastogenesis, we investigated the expression of WT1 transcripts in osteoclasts in bone tissues. An incidental and unexpected finding of this investigation was high level expression of WT1 antisense RNA in active osteoclasts in newborn rats. Northern blot and strand-specific RT-PCR analysis also detected high levels of WT1 antisense RNA in the mandible of newborn rats, in which numerous active osteoclasts are present. We further assessed the function of antisense WT1 RNA in an *in vitro* culture system for evaluating osteoclastogenesis.

Materials and Methods

Cell Cultures and Osteoclastogenesis

RAW-D cells, a sub-clone of the murine macrophage cell line RAW264, which have the ability to differentiate into osteoclast-like multinucleated cells,^{26,27} were maintained in α -minimal essential medium containing 10% fetal bovine serum. For osteoclastogenesis, RAW-D cells were cultured in 96-well plates (6.8×10^3 cells per well) in the presence of 20 ng/mL receptor activator of NF- κ B ligand (RANKL) and 1 ng/mL tumor necrosis factor- α . After 3 days, the cells were stained for tartrate-resistant acid phosphatase, and tartrate-resistant acid phosphatase-positive multinucleated cells were counted. Osteoclasts were also generated from rat bone marrow cultures as described previously.^{28,29} Bone marrow cells were obtained from tibia and femurs of male SD rats and cultured for 4 days in 24-well plates (1×10^6 cells per well) in the presence of 10^{-8} mol/L $1\alpha,25$ -dihydroxyvitamin D₃, 10 ng/mL RANKL, and 10% (v/v) heat-treated conditioned medium of rat osteoblastic cell line ROS17/2.8 (htROSCM).

Preparation of RNA Probes

Total RNA was prepared from adult spleen of SD rats or from rat bone marrow cultures for forming osteoclasts using

ISOGEN (Nippon Gene, Toyama, Japan). Complementary DNA fragments of rat WT1 (gb: NM_031534) were prepared from total RNA of adult spleen by RT-PCR using an RNA PCR kit (AMV) Ver2.1 (Takara, Kyoto, Japan) according to the manufacturer's protocol. Sequences of PCR primers were: WT1 sense primer: $5'_{316}$ GGTACAGCCGGG-GCCTGTGCG $_{335}^{3'}$; WT1 anti-sense primer: $5'_{593}$ GAGTAC-TGCTGCTCGCCAG $_{574}^{3'}$ (product size 278 bp). PCR products were ligated into the EcoRV site of pT7Blue-T vector (Promega, Madison, WI) (pWT1#1-2) followed by recloning into the BamHI/HindIII sites of pBluescript II KS(+). The direction of the insert DNA was determined by sequencing the plasmid DNA using M13-20 primer and M13 reverse primers. The plasmid was linearized with BamH I to prepare template DNA for WT1-sense probe or with Hind III to prepare template DNA for WT1-antisense probe. Digoxigenin-labeled sense and antisense RNA probes were prepared using an *in vitro* RNA synthesis kit (Nippon Gene, Toyama, Japan).

Animals and Treatment

Normal, fertilized SD rats were maintained in a specific pathogen-free environment; newborn rats were used for this research. All animal experiments were performed according to the guideline for Care and Use of Animals of Kyushu University.

In Situ Hybridization

Six-day-old SD rats were anesthetized and fixed by perfusion via the left ventricle with 4% paraformaldehyde in phosphate-buffered saline (PBS). Whole blocks of mandible were dissected from rats and further fixed for 12 hours in the same fixative solution at 4°C, washed in PBS, and then demineralized at 4°C for 3 days in Morse's solution [22.5% (v/v) formic acid, 10% (w/v) sodium citrate]. The tissues blocks were dehydrated and embedded in paraffin. Serial sections (4 μ m thick) were cut and transferred onto silane-coated glass slides. *In situ* hybridization was performed as described previously.^{30–32} After dewaxing in xylene and rehydration through an ethanol series, sections were rinsed in PBS and treated with 10 μ g/mL proteinase K (Sigma) for 10 minutes at 37°C, fixed in 4% (w/v) paraformaldehyde in PBS for 10 minutes, and then incubated with 0.2 mol/L HCl for 10 minutes. The sections were then treated twice in PBS containing 0.2% (w/v) glycine for 10 minutes, followed by rinsing in PBS. The sections were acetylated with 0.25% (v/v) acetic anhydride in 0.1 mmol/L triethanolamine (pH 8.0) for 10 minutes. Digoxigenin-labeled sense and antisense RNA probes corresponding to WT1 cDNA were prepared as described above and were used for strand-specific hybridization. Hybridized digoxigenin-labeled probes were detected using a DIG Nucleic Acid Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. After color development, the sections were counterstained with 0.2% (w/v) methyl green.

Northern Blot

Total RNA was extracted from rat mandible of 6-day-old SD rats using ISOGEN (Nippon Gene, Toyama, Japan) following the manufacturer's instructions. Samples of total RNA (7 µg) were denatured at 65°C for 5 minutes in 50% formamide and loaded on a 1% (w/v) agarose gel containing 6.6% (v/v) formamide. After electrophoresis, the RNA was transferred to nylon membranes (Genescreen; NEN Research Products, Boston, MA) in 10× standard saline citrate for 18 hours and then cross-linked with UV (254 nm, 120 mJ/cm²). The membrane was prehybridized in DIG-EasyHyb (Roche) for 30 minutes at 68°C, and hybridization was performed overnight with digoxigenin-labeled sense or antisense RNA probes corresponding to WT1 cDNA. After washing with 2× standard saline citrate containing 0.1% SDS, the membranes were incubated with blocking solution for 30 minutes at room temperature, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody for 30 minutes at room temperature. The membrane was washed, and the RNA was detected by a chemiluminescence reaction with CDP-Star (Roche). Blots were treated with stripping solution [50% (v/v) formamide, 25% SDS, 50 mmol/L Tris/HCl, pH 7.5] for 2 hours at 80°C and reused for another detection.

Strand-Specific RT-PCR

Total RNA was extracted from rat mandible of 6-day-old SD rats using ISOGEN (Nippon Gene), and cDNA was synthesized from 1 µg total RNA with AMV Reverse Transcriptase XL (Takara Bio, Kyoto, Japan) at 50°C for 1 hour. For the generation of strand-specific cDNA, either sense-specific (R1493) or antisense-specific (R284) primer was used. Sense- or antisense-specific cDNA was amplified with two different primer pairs (F1077/R1493 and F455/R708) using an RT-PCR kit (Takara Bio). PCR primer sequences to detect antisense RNA are listed in [Table 1](#).

Table 1 Primer Sequences Utilized in Strand-Specific RT-PCR

Primer name	Sequence
WT1 sense	
WT1sR1493 (R1493)	5'-TCTTGGAAGTCGGATGAAGATCCA-3'
WT1s1077F (F1077)	5'-TGACTTCAAGGACTGCGAGAGA-3'
WT1s1493R (R1493)	5'-TCTTGGAAGTCGGATGAAGATCCA-3'
WT1 antisense	
WT1asR284 (R284)	5'-CCTTCACCTTGCACTTCTCC-3'
WT1as455F (F455)	5'-GACACCCAGATGATGGGAA-3'
WT1as708R (R708)	5'-CGGAGTAACCGCATAGTGA-3'

Plasmid Construct for Antisense WT1 RNA and Transfection

The expression plasmid used to produce antisense RNA for WT1 was constructed from the pMKITneo vector (kindly provided by Dr. Maruyama of Tokyo Medical and Dental University, Tokyo, Japan) having the SRα promoter upstream of the multiple cloning site. For encoding WT1 antisense RNA, the SpeI/EcoRI fragment excised from pWT#1-2 plasmid (see [Preparation of RNA Probe](#)) was cloned into SpeI/EcoRI site, down-stream of SRα promoter of pMKITneo. Accuracy of the construct was confirmed by nucleotide sequencing. Plasmid DNA was purified with a Plasmid Purification Kit (Qiagen, Valencia, CA), and transfected into RAW-D cells using the Nucleofector System (Amaxa Biosystems, Gaithersburg, MD). Briefly, 3 × 10⁶ cells were transfected with 0.75 to 2 µg plasmid DNA in the presence of Nucleofector reagents according to the manufacturer's protocol. The numbers of tartrate-resistant acid phosphatase-positive multinucleated cells were counted after 3 days of culture in the presence of RANKL. Data were analyzed by Student's *t*-test. At initiation and end of culture, total proteins were extracted for Western blotting.

Real-Time PCR Analysis

Total RNA was extracted using Isogen (Nippon Gene). Total RNA was reverse transcribed with a PrimeScript RT-PCR kit (Takara) by use of random primers. Real-time PCRs were performed by using SYBR Primer EX Taq (Takara Bio) with a StepONEplus real-time PCR system (Applied Biosystems). The following primers were used for real-time RT-PCR analysis: mouse *Ctsk* (cathepsin K) sense, 5'-CTCTCGGC-GTTTAATTTGGGAG-3', antisense, 5'-CCCTGGTTCTTG-ACTGGAGTAACG-3'; mouse *Zbtb7a* (LRF) sense, 5'-GA-GAAGAAAATCCGGGCCAAG-3', antisense, 5'-GCAGC-TATCGCACTGGTATGG-3'; mouse *Nfatc1* sense, 5'-TCAT-CGGCGGGAAGAAGATG-3', antisense, 5'-AATTTGGC-TTGACAGGTCC-3'; mouse *Fos* sense, 5'-GAAGGGG-CAAAGTAGAGCAG-3', antisense, 5'-GTTGATCTGTCT-CCGCTTGG-3'; mouse *Gapdh* sense, 5'-TGCACCACC-AACTGCTTAG-3', antisense, 5'-GGATGCAGGGATGAT-GTTC-3'. mRNA levels were normalized to *Gapdh* expression.

Western Blot

Cells were lysed in lysis buffer containing 1% (v/v) Triton X-100, 0.5% (v/v) NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), and protease inhibitor cocktail (Sigma, St. Louis, MO). Aliquots of lysates, containing equal amounts of lysate proteins, were separated by 10% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane. After blocking in 5% nonfat dry milk in tris-buffered saline, the membrane was probed with rabbit anti-WT1 antibody

(Santa Cruz Biotechnology, Dallas, TX), followed by rinsing in tris-buffered saline. Proteins were detected with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK). As a loading control, the membrane was stripped, reprobed with anti-actin antibody (Santa Cruz Biotechnology), and visualized by ECL Plus kit (Amersham Biosciences).

Results

Expression of WT1 Protein during Osteoclastogenesis

Immunoblotting analysis showed that RAW-D osteoclast precursor cells expressed high levels of WT1 protein when the cells were not under stimulation by osteoclastogenic factors. However, the level of WT1 protein in osteoclast precursors was markedly reduced when cells were stimulated with RANKL and tumor necrosis factor- α to induce osteoclast formation (Figure 1). Murine spleen cells (positive control), which are known to contain abundant hematopoietic progenitor cells, expressed high levels of WT1 (Figure 1). High levels of WT1 protein expression were also observed in rat bone marrow cells, which contain numerous hematopoietic progenitors, including osteoclast precursors.

Figure 1 Expression of WT1 protein during osteoclastogenesis. Total proteins were extracted from RAW-D cells and rat bone marrow cells cultured for forming osteoclasts, as described in *Materials and Methods*. Total proteins were also isolated from murine spleen cells. **A:** Equal amounts of proteins were subjected to Western blot analysis with anti-WT1 antibody or anti-actin antibody. Lane 1 to 4 (from the left): RAW-D cells cultured for 0, 1, 2, 3 days for forming osteoclasts. Lane 5: Mouse spleen cells as positive control for WT1 expression (mouse spleen). Lanes 6 and 7: Rat bone marrow cells cultured (day 0 and day 4) for forming osteoclasts, as described in *Materials and Methods*. Actin is used as the loading control. **B:** The ratio of WT1/actin was calculated by NIH Scion image analyzer for each lane. Experiments were performed three times with similar results.

Marked suppression of WT1 protein expression was detected when rat bone marrow cells were stimulated to differentiate into osteoclasts (Figure 1). These data demonstrate that the expression of WT1 protein is markedly down-regulated during the process of osteoclast differentiation.

High Expression of Antisense WT1 RNA in Active Osteoclasts *in Vivo*

To determine the expression of WT1 mRNA *in vivo*, we examined the expression of WT1 mRNA in the mandibles of newborn rats. Low levels of WT1 mRNA were detected in osteoclasts (Figure 2A). Unexpectedly, high expression of WT1 antisense RNA was detected in osteoclasts located on the surface of the alveolar bone adjacent to incisor tooth germ; these cells were presumed to be migrating between bone trabeculae (Figure 2B). No WT1 mRNA was detected in active osteoclasts (Figure 2, C and E), but marked expression of WT1 antisense RNA was detected in osteoclasts engulfing the tip of bone trabeculae (Figure 2, D and F). These cells may have been actively resorbing bone. These data show that osteoclasts express high levels of WT1 antisense RNA.

Sense Transcripts Anti sense Transcripts

Figure 2 Extremely high expression of antisense WT1 RNA in osteoclasts in the mandible of newborn rats revealed by RNA *in situ* hybridization. WT1 antisense probe (**A**, **C**, and **E**) or sense probe (**B**, **D**, and **F**) was hybridized with tissue sections of mandible. Purple shows the transcripts. Nuclear staining was performed with methyl-green after detecting the transcripts. **E** and **F** are the high magnification view of **C** and **D** (asterisks), respectively. The left column and the right column are the serial sections (**A** and **B**, **C** and **D**, **E** and **F**, respectively). Arrows show the position of the typical osteoclasts. Scale bars: 50 μ m (**A–D**); 10 μ m (**E** and **F**). b, bone; i, incisor tooth germ; tb, trabecular bone.

Figure 3 Detection of WT1 antisense RNA in mandible of newborn rats by Northern blotting and strand-specific RT-PCR. **A:** Northern blotting: Total RNAs were extracted from mandible of newborn rats. Equal amount of RNAs was subjected to agarose gel electrophoresis followed by transfer to nylon membranes and subjected to Northern blot analysis by use of the strand-specific RNA probes. **Left panel:** Antisense RNA transcripts detected by the sense RNA probe for WT1. **Right panel:** Sense RNA transcripts (mRNA) detected by antisense RNA probes. Position of the origin (ori) and the size of ribosomal RNAs (28S and 18S) are shown. **B:** Strategy for detecting antisense RNA using strand-specific primers. Details were described in *Materials and Methods*. GenBank accession number of WT1 rat RNA is NM_031534 (<http://www.ncbi.nlm.nih.gov/nucore>). **C:** Strand-specific RT-PCR: RNAs from mandible of newborn rats were also subjected to the strand-specific RT-PCR, as described in *Materials and Methods*. Left lane: size marker. Middle lane: antisense RNAs. Right lane: sense RNAs (mRNAs).

We confirmed the expression of WT1 antisense RNA in mandible by Northern blot analysis and strand-specific RT-PCR. The Northern blot data showed expression of antisense WT1 RNA of multiple sizes in mandible of newborn rats (Figure 3A), whereas only a faint signal corresponding to WT1 sense RNA (mRNA) was detected by Northern blot (Figure 3A). Strand-specific RT-PCR of total RNA obtained from mandible of newborn rats using a specific set of primers (Figure 3B and Table 1) detected a 254-bp PCR product, indicating the expression of antisense WT1 RNA (Figure 3C).

Function of Antisense WT1 RNA in Osteoclastogenesis

To assess the role of WT1 protein and the function of WT1 antisense RNA expressed in cells of the osteoclast lineage, an

expression plasmid designed to express WT1 antisense RNA (Figure 4A) was transfected into RAW-D cells. When these transfected cells were induced to form osteoclasts, osteoclastogenesis was markedly enhanced by overexpression of WT1 antisense RNA compared to empty vector controls (Figure 4, B and C). We confirmed the suppression of WT1 protein synthesis in transfected cells by Western blot (Figure 4D). The protein levels of WT1 were markedly decreased in RAW-D cells transfected with the antisense WT1 RNA expression plasmid, but transfection of empty vector had no effect on the expression of WT1 protein (Figure 4D). These data indicate that WT1 antisense RNA has the ability to stimulate osteoclastogenesis by suppressing WT1 protein expression.

To know the function of WT1 antisense RNA in the expression of several molecules related to osteoclast

Figure 4 Marked stimulation of osteoclastogenesis by overexpressing WT1 antisense RNAs. RAW-D cells were transfected with the plasmid DNA expressing WT1 antisense RNA (WT1-AS) or with empty vector (control) and further cultured in the presence of 20 ng/mL receptor activator of NF- κ B ligand and 1 ng/mL of tumor necrosis factor (TNF)- α . **A**: Schematic demonstration of the construct expressing antisense WT1 RNA. Preparation of the construct was performed as described in *Materials and Methods*. **B** and **C**: Marked elevation of osteoclastogenesis by transfecting WT1-AS construct. After 3 days of culture of the transfected RAW-D cells, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP); **(B)** and number of TRAP-positive multinucleated cells (**arrows**) was counted **(C)**. Statistical analysis was performed using paired Student's *t*-test. **D**: Suppression of WT1 protein by transfection of a construct expressing WT1 antisense RNA. Total cell lysates were prepared from RAW-D cells transfected with the empty vector (left lane: Cont.) or WT1 antisense RNA-expressing vector (right lane: WT1-AS), and equal amounts of proteins were subjected to Western blot analysis with anti-WT1 and anti-actin antibodies (**left panel**). Actin was used as a loading control. The ratio of WT1/actin was calculated by NIH Scion image analyzer (**right panel**). Data are expressed as means $\pm$ SEM from quadruplicate cultures (**C**). $^{**}P < 0.01$. Cont., control.

differentiation, we have examined the expression of *Ctsk*, *Nfatc1*, *Zbtb7a* (gene for OCZF/LRF), and *cfos* in RAW-D cells transfected with expression plasmid, which express WT1 antisense RNA. **Figure 5** shows the effects of WT1 antisense RNA expression on osteoclastogenesis when transfected RAW-D cells were stimulated with RANKL for 3 days. Although WT1 antisense RNA did not significantly affect the expression of *Nfatc1*, *cfos*, and *Zbtb7a*, it significantly stimulated expression of *Ctsk* gene. These data suggest that WT1 antisense RNA has a regulatory role in gene expression required for osteoclasts, at least in the late stage of osteoclast differentiation.

Discussion

In the current study, we have found high expression of WT1 antisense RNA in mature osteoclasts. Our functional studies suggested that WT1 antisense RNA has the ability to augment osteoclastogenesis by inhibiting WT1 protein expression. We have detected high expression of WT1 protein in an osteoclast precursor cell line and in bone marrow cells before induction of osteoclastogenesis. After osteoclast induction, expression of WT1 protein was markedly suppressed. A possible molecular mechanism mediating suppression of WT1 protein expression during

osteoclastogenesis was suggested by the RNA *in situ* hybridization analysis, which showed low levels of WT1 mRNA in multinucleated osteoclasts migrating between bone trabeculae and undetectable WT1 mRNA in activated osteoclasts adhered to the tip of bone trabeculae, but high expression of WT1 antisense RNA in these cells. Several reports have been suggested that WT1 antisense RNA regulates the expression of WT1 protein. Moorwood et al³³ previously demonstrated that WT1 antisense transcripts colocalized with WT1 protein and mRNA in fetal kidney podocytes and glomeruli, consistent with a role for WT1 antisense RNA in positive regulation of WT1 expression. In contrast, Dallosso et al³⁴ showed that WT1 antisense RNA was able to suppress WT1 protein levels in an *in vitro* model. In their model, WT1 antisense RNA forms a duplex with mRNA, which blocks WT1 protein synthesis. Our results are similar, and indicate that the WT1 antisense RNA regulates osteoclastogenesis through down-regulating WT1 protein synthesis during osteoclastogenesis. A high level of WT1 antisense RNA in active osteoclasts could ensure complete suppression of WT1 protein expression, which might avoid loss of differentiation capacity of osteoclasts (**Figure 6**). Osteoclastogenesis is controlled by several transcription factors, including c-fos,³⁵ NFATc1,³⁶ OCZF/LRF,^{29,37,38} among others. We have examined the regulatory function of WT1 antisense RNA in the expression of

Figure 5 Significant stimulation of *Ctsk* gene expression by WT1 antisense RNA. RAW-D cells were transfected with 1.5 μ g of plasmid designed to express WT1 antisense RNA (WT1-AS) or empty vector (Cont.) followed by being stimulated with 40 ng/mL receptor activator of NF- κ B ligand for 68 hours. Total RNA was extracted and subjected to real-time RT-PCR, as described in *Materials and Methods*. Experiments were performed three times with similar results. $n = 4$. ** $P < 0.01$ versus control. Cont., control.

these transcription factors in late stage of osteoclastogenesis. Although statistically significant effects were not observed, there exists a tendency that WT1 antisense RNA enhanced expression of *Nfatc1* and *fos*. If we examined the expression of these transcription factors in earlier stages of osteoclast

differentiation, we might successfully detect the significant induction of these transcription factors. It will be important to elucidate the functional relationship between these transcription factors and WT1 antisense RNA in the induction and regulation of osteoclastogenesis. Further extensive studies are required to elucidate the molecular mechanism of the action of WT1 antisense RNA on gene expression essential for osteoclast differentiation. In these analyses, we have detected a significant stimulation of *Ctsk* gene expression by WT1 antisense RNA. As the *Ctsk* gene product, cathepsin K, is an essential cysteine protease required for bone resorption, induction of WT antisense RNA could have a supportive role in osteoclastic bone resorption. Extensive investigation of the relationship between the expression of WT1 antisense RNA and the activity of osteoclasts can open new avenues regarding the regulation of osteoclast activation by the antisense RNAs.

It has been shown that miR-21 induced by RANKL is essential for osteoclastogenesis, and that silencing of miR-21 expression impairs osteoclastogenesis.³⁹ Recently, it has been reported that various miRNAs, including miR-29,⁴⁰ miR-31,⁴¹ miR-124,⁴² miR-125a,⁴³ miR-148a,⁴⁴ miR-155,⁴⁵ and miR-223,⁴⁶ are involved in the regulation of osteoclastogenesis. Krzeszinski et al⁴⁷ showed that miR-34a is a suppressor of osteoclastogenesis and bone resorption, and suppresses the bone metastatic niche reducing metastasis. Several reports have described the expression of IGF2-receptor antisense RNAs, which are thought to be involved in genomic imprinting.^{48,49} Our current findings reported herein demonstrate a possible mechanism for fine regulation related to maintenance of osteoclast differentiation states by WT1 antisense RNAs.

Dallosso et al³⁴ defined the precise promoters of WT1 antisense transcripts in intron 1 of the murine *Wt1* gene, and found several antisense RNA species in kidney and ovary of different sizes that were formed through alternative splicing. In our current Northern blot experiments, we detected several WT1 antisense RNAs of different sizes, which probably reflect alternatively spliced WT1 antisense transcripts. We continue to pursue elucidation of the molecular details of these WT1 antisense RNAs detected in active osteoclasts in mandible of newborn rats. WT1 protein is highly expressed in hematopoietic progenitors and bone marrow cells before differentiation into osteoclasts. Thus, regulation of antisense RNA may provide an avenue toward development of novel therapies for controlling pathological bone resorption.

Figure 6 Schematic illustration of a hypothetical role of WT1-antisense RNA in the fine regulation of osteoclast differentiation. Macrophages and osteoclasts differentiate from a common progenitor. High expression of WT1 protein detected in osteoclast progenitors is down-regulated during osteoclast differentiation. WT1 antisense RNA (WT1-ASRNA) expressed in cells in the osteoclast lineage can have a regulatory role in the induction of osteoclastogenesis from progenitors and a regulatory role in maintaining differentiation status in mature osteoclasts.

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T.K. directed the project; Y.-J.L., A.K., and T.K. wrote the manuscript; T.K. performed the *in situ* hybridization; Y.-J.L., A.K., and Y.K.-N. performed gene expression analysis; all authors analyzed data.

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