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# 研究成果の刊行物・別刷

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# Vascular endothelial growth factor-A is a survival factor for nucleus pulposus cells in the intervertebral disc

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ABSTRACT

The intervertebral disc (IVD) is composed of two avascular tissue types, the nucleus pulposus (NP) and the annulus fibrosus (AF). IVDs is the largest avascular tissue in the human body, however, how these tissues are maintained without a blood supply is poorly understood. Here we show that vascular endothelial growth factor-A (VEGF-A) is highly expressed in NP and that VEGF-A plays a role in NP survival. High VEGF-A expression in NP was detected by microarray analysis, and NP was positive for the hypoxic probe pimonidazole and hypoxia-responsive genes. VEGF-A expression in NP was promoted by hypoxic conditions *in vitro*. NP cells also expressed the membrane-bound VEGF receptor-1 (VEGFR-1), and the number of apoptotic cells in cultured cell model of NP increased following treatment with VEGFR-1-Fc, which traps VEGF-A in NP. These results indicate that NP is a hypoxic tissue, and that VEGF-A functions in NP survival in an autocrine/paracrine manner.



The intervertebral disc (IVD) is located between vertebral bodies and is composed of two distinct tissues: a gelatinous center known as the nucleus pulposus (NP) and surrounding coaxial lamellae that form the annulus fibrosus (AF). NP is an avascular tissue, and NP avascularity is crucial for NP homeostasis and function. NP plays essential roles in flexibility and stability of the spine, which decrease with aging, suggesting that NP homeostasis may be downregulated in an age-dependent manner or that the NP undergoes degeneration with aging.

NP is rich in chondrogenic extracellular-matrix (ECM) proteins, which maintain tissue integrity. Recent studies indicate that several genes encoding ECM proteins function in the etiology and pathogenesis of IVD degeneration, and that these proteins maintain IVD homeostasis in humans and mice [1–3].

VEGF-A has strong angiogenic activity and specific mitogenic and chemotactic actions on endothelial cells [4]. VEGF-A synthesis is increased several fold by hypoxia [5]. Vascular endothelial cells highly express both VEGF receptor-1 (VEGFR-1) and VEGFR-2 [6]. Non-endothelial cells, including monocytes and hematopoietic stem cells, reportedly express VEGFR-1 or VEGFR-2, respectively [7–9]. A soluble form of VEGFR-1 (sVEGFR-1), a VEGFR-1 splice variant, has been shown to have potent anti-angiogenic activity in cornea [10]. On the other hand, VEGFR-1-mediated signaling through the membrane-bound form of VEGFR-1 (mbVEGFR-1) reportedly plays a role in pathological conditions, including carcinogenesis and inflammatory disease [11,12].

Here, we demonstrate that use microarray, RT-PCR and immunohistochemical analysis to show that NP cells express both VEGF-A and mbVEGFR-1, and that VEGF-A acts as a survival factor in NP in an autocrine/paracrine manner. NP is a hypoxic probe, pimonidazole, positive and hypoxia-responsive gene expressing hypoxic tissue, and VEGF-A expression is promoted by hypoxia in NP.

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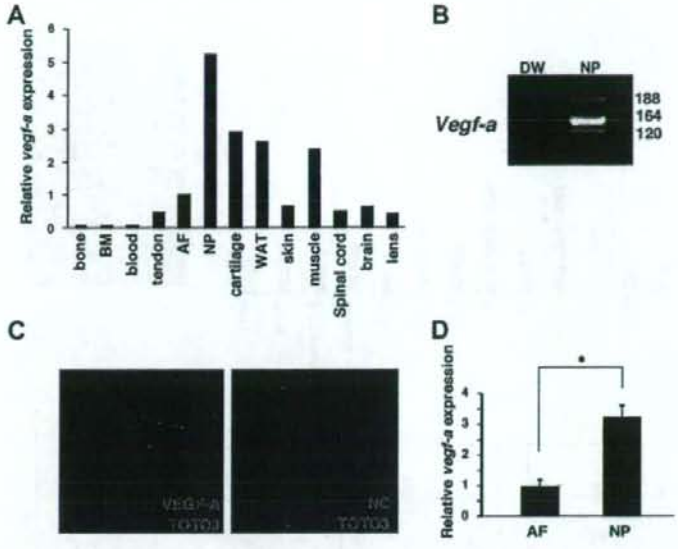


**Fig. 1.** VEGF-A expression is high in NP of the intervertebral disc (IVD). (A) Microarray analysis of 13 tissues including NP from Wistar rats. AF, annulus fibrosis; NP, nucleus pulposus; BM, bone marrow; WAT, white adipose tissue. (B) *Vegf-A* splice variants VEGF120, 164 and 188 were detected in rat NP by RT-PCR analysis. (C) IVD dissected from 8-week-old rats was stained with rabbit anti-VEGF antibody (VEGF-A) followed by Alexa Fluor488-conjugated anti-rabbit IgG antibody and observed by confocal microscopy. TOTO3 was used as a counter-stain for nuclei. NC, no primary antibody control. (D) Semi-quantitative real-time PCR analysis of *Vegf-A* mRNA expression in human samples. Data are mean relative ratios ± SD of *Vegf-A*/*β-actin* mRNA expression in NP compared with that of AF ($^*P < 0.01$).

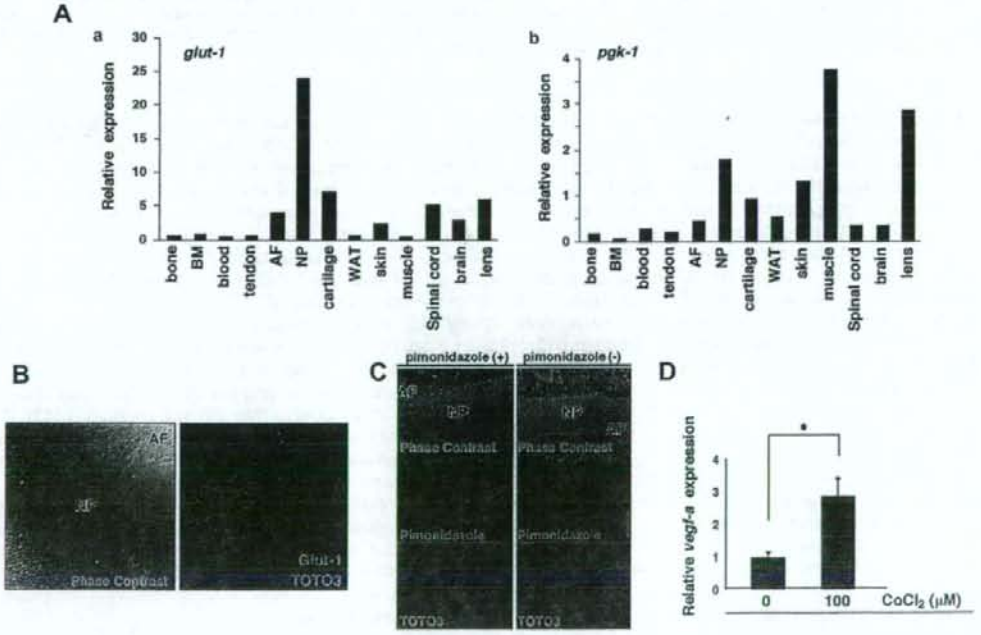


Fig. 2. VEGF-A expression in NP is promoted by hypoxic conditions. (A) Microarray analysis of hypoxia-responsive genes (a) *glut*-1 and *pgk*-1 (b). (B) IVD sections of 8-week-old rats were stained by rabbit anti-Glut-1 antibody followed by Alexa Fluor488-conjugated anti-rabbit IgG and observed under confocal microscope. TOTO3 served as counter-stain for nuclei. (C) The hypoxic probe, pimonidazole, was injected intravenously into 8-week-old mice and 3 h later IVDs were dissected from pimonidazole-injected (left panels) or -uninjected (right panels) mice. Sections were stained with mouse anti-pimonidazole antibody, followed by Alexa Fluor488-conjugated anti-mouse IgG, and observed by confocal microscopy. (D) NP cells were isolated from 8-week-old rats and cultured in the presence or absence of 100 μM $CoCl_2$. After 24 h, *Vegf-A* mRNA expression in NP was analyzed by semi-quantitative real-time PCR. Data are mean relative ratios ± SD of *Vegf-A*/*β-actin* mRNA expression in $CoCl_2$ (100 μM)-treated NP compared with non-treated (0 μM) NP ($^*P < 0.01$).

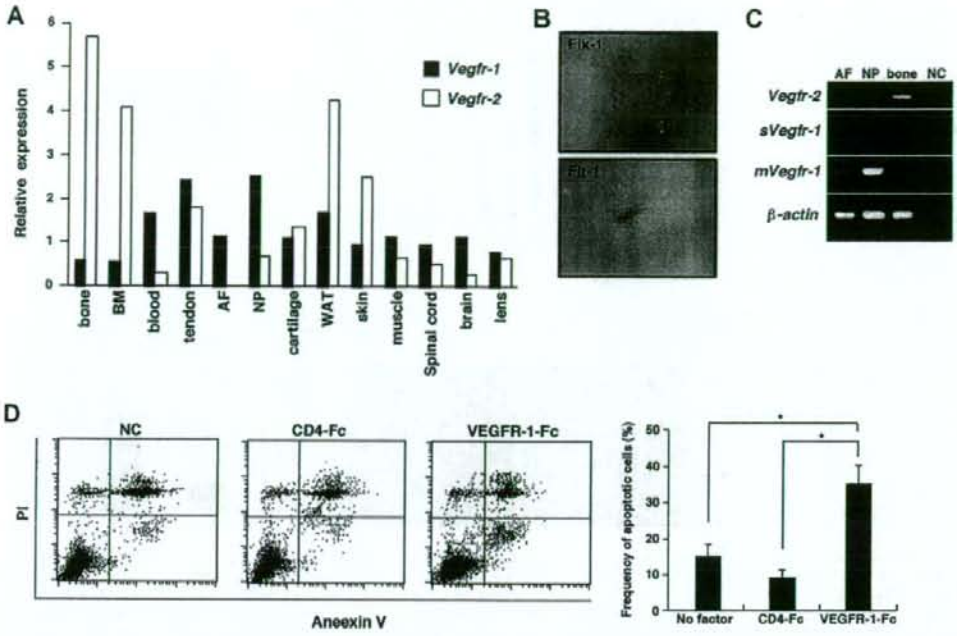


**Fig. 4.** Membrane-bound VEGFR-1 (mbVEGFR-1) is expressed in NP. (A) *Vegfr-1* and *Vegfr-2* expression was analyzed by microarray analysis. AF, annulus fibrosis; NP, nucleus pulposus; BM, bone marrow; WAT, white adipose tissue. (B) LacZ staining of NP derived from *Vegfr-2*$^{lacZ/+}$ (left panel) and *Vegfr-1*$^{lacZ/+}$ (right panel) mice. (C) *Vegfr-2*, soluble *Vegfr-1* (*sVegfr-1*) and membrane-bound *Vegfr-1* (*mbVegfr-1*) expression was analyzed in AF, NP and bone by RT-PCR. (D) NP cells isolated from 8-week-old rats were suspended in sodium alginate and cultured in serum free medium with or without 10 μg/ml sVEGFR-1-Fc or CD4-Fc for 48 h. Cells were then stained with FITC-conjugated annexin-V and propidium iodide (PI) and analyzed by flow cytometry using FACS Calibur. Apoptotic cells were detected as annexin-V-positive/PI-negative. Upper panels, representative data; lower panels, mean frequency ± SD of apoptotic cells ($^*P < 0.01$).

blood supply. Here we demonstrate that VEGF-A is induced by hypoxia in NP and that VEGF-A functions in NP as a survival factor. Since mbVEGFR-1, a VEGF-A receptor with 10-fold higher affinity for VEGF-A compared with VEGFR-2, is also expressed in NP, vascular invasion may be inhibited in part by an autocrine trap consisting of VEGF-A-expressing cells that also express VEGFR-1.

Here we performed microarray analysis in 13 tissues including five avascular ones, namely, NP, AF, cartilage, tendon and lens. Interestingly, we did not identify common molecules highly expressed in avascular tissues, suggesting that mechanisms underlying maintenance of avascularity are tissue-specific. Recently Yoshioka et al. reported that chondromodulin-I functions to prevent angiogenesis in avascular cardiac valves [18], although such a function has not been detected in cartilage, which also expresses chondromodulin-I [19,20]. On the other hand, Ambati et al. report that cornea, another avascular tissue, exclusively expresses sVEGFR-1 [10]. In NP, aggrecan reportedly inhibits endothelial cell adhesion and cell migration [21]. *Aggrecan* expression is high in NP, AF and cartilage but low in tendon and lens among avascular tissues that we analyzed (data not shown), further supporting the idea that mechanisms underlying maintenance of avascularity are tissue-specific.

VEGF-A expression is promoted by hypoxic conditions in NP. In the hypoxic lens, expression of hypoxic response genes such as *glut-1* and *pgk-1* was observed [22]. However, *Vegf-A* expression in lens is much lower than that of NP (data not shown). These results suggest that VEGF-A expression is not simply promoted by hypoxic conditions but is regulated by tissue-specific factors other than HIF-1α. Further studies are needed to clarify how VEGF-A expression is specifically regulated in NP.

There are two types of tyrosine kinase VEGF-A receptors: VEGFR-1 and VEGFR-2. We found that both VEGF-A and mbVEGFR-1 were expressed in NP, while VEGFR-2 was not (Figs. 1 and 2). These results suggest that VEGF-A signaling can be transduced through VEGFR-1 in an autocrine or a paracrine manner in NP. VEGF-A is known to transduce a migration signal in macrophages and a survival signal in tumors and hematopoietic stem cells [9,12]. We found that NP cells express both VEGF-A and VEGFR-1 and that treatment of NP cells with the VEGFR-1-Fc, a VEGF-A antagonist, induced apoptosis in NP cells, suggesting that the VEGF-A/VEGFR-1 cascade mediates an anti-apoptotic function in NP. Similar to NP, several tumor cells express both VEGF-A and VEGFR-1, and autocrine interactions of VEGF-A and VEGFR-1 function to resist apoptosis under hypoxic conditions [12]. On the other hand, hematopoietic stem cells express both VEGF-A and VEGFR-2, and genetic ablation of VEGF-A in hematopoietic stem cells reduces cell survival, suggesting that autocrine interaction of VEGF-A and VEGFR-2 is crucial for cell survival [9]. Apoptotic cells in NP reportedly increase with aging [23], suggesting that NP degeneration is associated with aging. We have shown here that VEGF-A expression in NP is downregulated with age. Thus regulation of VEGF-A expression in NP may be a good target to prevent age-associated degeneration.

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*Original article*

# Japanese Orthopaedic Association Back Pain Evaluation Questionnaire. Part 3. Validity study and establishment of the measurement scale

## Subcommittee on Low Back Pain and Cervical Myelopathy Evaluation of the Clinical Outcome Committee of the Japanese Orthopaedic Association, Japan

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**Abstract**

***Background.*** The Japanese Orthopaedic Association decided to revise the JOA score for low back pain and to develop a new outcome measure. In February 2002, the first survey was performed with a preliminary questionnaire consisting of 60 evaluation items. Based on findings of that survey, 25 items were selected for a draft of the JOA Back Pain Evaluation Questionnaire (JOABPEQ). The second survey was performed to confirm the reliability of the draft questionnaire. This article further evaluates the validity of this questionnaire and establishes a measurement scale.

***Methods.*** The subjects of this study consisted of 355 patients with low back disorders of any type (201 men, 154 women; mean age 50.7 years). Each patient was asked to fill in a self-administered questionnaire. Superficial validity was checked in terms of the completion rate for filling out the entire questionnaire. Factor analysis was then performed to evaluate the validity of the questionnaire and establish a measurement scale.

***Results.*** As a result of the factor analysis, 25 items were categorized into five factors. The factors were named based on the commonality of the items: social function, mental health, lumbar function, walking ability, and low back pain. To establish a measurement scale for each factor, we determined the coefficient for each item so the difference between the maximum factor scores and minimum factor scores was approximately 100. We adjusted the formula so the maximum for each factor score was 100 and the minimum was 0.

***Conclusions.*** We confirmed the validity of the JOA Back Pain Evaluation Questionnaire and est ablished a measurement scale.

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## Introduction

The evaluation criteria were based on physiological, biological, and anatomical outcome measure results of the Japanese Orthopaedic Association (JOA) score for low back pain.[1] The criteria include laboratory values, physiological findings, and imaging findings. These findings are significant for doctors but have little meaning for patients. From a patient's perspective, the presence of a symptom or its degree and functional condition

**Table 2.** Distribution of the severity evaluated by the current JOA scoring system and finger-floor distance ($n$ = 355)

| Parameter | No. |
|---|---|
| Straight-leg raising (SLR) test | |
| Normal | 183 |
| 30°–70° | 130 |
| <30° | 42 |
| Motor function | |
| Normal | 182 |
| Slight weakness (MMT good) | 126 |
| Severe weakness (MMT less than good) | 47 |
| Sensory function | |
| Normal | 127 |
| Slight disturbance | 162 |
| Severe disturbance | 66 |
| Bladder function | |
| Normal | 315 |
| Mild dysuria | 36 |
| Severe dysuria | 4 |
| Finger-to-floor distance (cm) | |
| to −15 | 1 |
| −14 to −5 | 12 |
| −4 to 4 | 69 |
| 5 to 14 | 73 |
| 15 to 24 | 69 |
| 25 to 34 | 43 |
| 35 to 44 | 25 |
| 45 to 54 | 30 |
| 55 to 64 | 6 |
| 65 to 74 | 4 |
| Not measurable | 14 |
| Total number | 355 |

JOA, Japanese Orthopaedic Association; MMT, manual muscle testing

specific. There was no marked difference in the distribution of the severity levels between the 451 patients who were initially recruited and the 355 who were finally analyzed.

*Superficial validity*

Superficial validity was checked in terms of the completion rate for filling out the questionnaire. Regarding the distribution of responses for each item, it was judged that none of the questions was too difficult to answer because the highest rate of nonrespose was 1.8%. With regard to deflection of an answer, the highest rate (78.3%) was concentrated on "yes" responses to question 1–14, although this was judged not to be inappropriate. Therefore, the distribution was not skewed, which would indicate "floor and ceiling" effects (Table 3).

*Factor analysis*

First, we tried to extract some observed variables from 25 items by the Maximum Likelihood Method. It was found that the eigenvalue was >1.0 for five items, and the accumulative contribution ratio until the fifth factor was 53.1% (Table 4).

Next, we performed orthogonal rotation by the direct oblimin method. The results are shown in Table 5. Each item was categorized into five factors: Four items (Q2-6, Q2-5, Q1-2, Q2-4) related to factor 1; seven items (Q2-8, Q2-7, Q2-11, Q1-13, Q2-9, Q2-10, Q2-1) related to factor 2; six items (Q1-9, Q1-6, Q2-3, Q1-8, Q1-5, Q1-4) related to factor 3; five items (Q1-10, Q2-4, Q1-12, Q1-14, Q2-2) to factor 4; and the last four items to factor 5. Although factor loading was <0.30 in three items (Q1-4 to factor 3, Q2-2 to factor 4, Q1-11 to factor 5), we adopted all of them for the reason that the question itself was important for the factor or the number of questions in each factor needed to be more than four.

Factor names were determined based on the commonality of the items that showed a large value on factor loading: factor 1, social function (four items); factor 2, mental health (seven items); factor 3, lumbar function (six items); factor 4, walking ability (five items); and factor 5, low back pain (four items).

*Measurement scale*

To establish a measurement scale for each factor, we determined the size of the coefficient for each item so the difference between the maximum factor scores and minimum factor scores was approximately 100 (Table 6). When a coefficient became a negative numerical value, we changed the coefficient to a positive numerical value by reversing the order of the answer choice. We adjusted the formula so the maximum for each factor score was 100 and the minimum was 0 (see Appendix 2).

## Discussion

It is considered ideal for the outcome measure to evaluate patients from various perspectives, such as dysfunction, disability, handicap, and psychological problem. The outcome measure should be patient-oriented, and its reliability and validity should be confirmed by statistical analysis. However, the current JOA score does not include subjective evaluations and does not meet such requirements. We developed a new questionnaire, JOABPEQ, specifically to evaluate low back pain. It is patient-oriented and mainly based on recognizing problems with activities of daily living. We categorized 25 questions into five factors; each factor is then scored up to 100 points using the measurement scale. The factors are then evaluated separately. The point is to be aware that it is meaningless and inadequate to total

**Table 6.** Coefficient for each item of the formula for measurement scale

| Item | 1 Social function | 2 Mental health | 3 Lumbar function | 4 Walking ability | 5 Low back pain |
|---|---|---|---|---|---|
| Q1-1 | | | | | 20 |
| Q1-2 | 2 | | | | |
| Q1-3 | | | | | 20 |
| Q1-4 | | | 10 | | |
| Q1-5 | | | 10 | | |
| Q1-6 | | | 20 | | |
| Q1-7 | | | | | 20 |
| Q1-8 | | | 10 | | |
| Q1-9 | | | 30 | | |
| Q1-10 | | | | 30 | |
| Q1-11 | | | | | 10 |
| Q1-12 | | | | 20 | |
| Q1-13 | | 3 | | | |
| Q1-14 | | | | 10 | |
| Q2-1 | | -4 | | | |
| Q2-2 | | | | 10 | |
| Q2-3 | | | 20 | | |
| Q2-4 | 4 | | | 30 | |
| Q2-5 | 6 | | | | |
| Q2-6 | 10 | | | | |
| Q2-7 | | 6 | | | |
| Q2-8 | | 6 | | | |
| Q2-9 | | -3 | | | |
| Q2-10 | | -3 | | | |
| Q2-11 | | 3 | | | |

the five factors' scores; rather, they should be treated by nonparametric analysis. The reliability of the questionnaire including 25 items for the JOABPEQ was confirmed in Part 2 of this project. The validity of the questionnaire was evaluated using factor analysis, and the measurement scale was established in Part 3 of this study. Further studies must be performed to confirm the responsiveness of the calculations of the severity score.

## Conclusions

We confirmed the validity of the JOA Back Pain Evaluation Questionnaire (JOABPEQ) and established a measurement scale.

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## Appendix 1. Items selected for the draft of a JOABPEQ document

With regard to your health condition during the last week, please choose the item number among the answers for the following questions that best applies as your condition varies depending on the day or time. Circle the item number when your condition is at its worst.

**Q1-1** To alleviate low back pain, you often change your posture.
1) Yes
2) No

**Q1-2** Because of low back pain, you do not do any routine housework these days.
1) No
2) Yes

**Q1-3** Because of low back pain, you lie down more often than usual.
1) Yes
2) No

**Q2-11** Do you feel your health will get worse?
1) Very m uch so
2) A little bit at a time
3) Sometimes yes and sometimes no
4) Not very much
5) Not at all

## Appendix 2. Measurement scale for JOABEPQ

Social life function
('Q1-2' × 2 + 'Q2-4' × 4 + 'Q2-5' × 6 + 'Q2-6' × 10 − 22) × 100 ÷ 74

Mental health
('Q1-13' × 3 + 'Q2-1' × 4 + 'Q2-7' × 6 + 'Q2-8' × 6 + 'Q2-9' × 3 + 'Q2-10' × 3 + 'Q2-11' × 3 − 28) × 100 ÷ 103

Lumbar function
('Q1-4' × 10 + 'Q1-5' × 10 + 'Q1-6' × 20 + 'Q1-8' × 10 + 'Q1-9' × 30 + 'Q2-3' × 20 − 100) × 100 ÷ 120

Walking ability
('Q1-10' × 30 + 'Q1-12' × 20 + 'Q1-14' × 10 + 'Q2-2' × 10 + 'Q2-4' × 30 − 100) × 100 ÷ 140

Low back pain
('Q1-1' × 20 + 'Q1-3' × 20 + 'Q1-7' × 20 + 'Q1-11' × 10 − 70) × 100 ÷ 70

blood

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# PIAS3 negatively regulates RANKL-mediated osteoclastogenesis directly in osteoclast precursors and indirectly via osteoblasts

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