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H. 知的財産権の出願・登録状況

1. 特許取得

骨粗鬆症の診断および/または骨粗鬆症骨折リスクの予測の方法

2. 実用新案登録

なし

3. その他

なし

Ⅱ. 研究成果の刊行に関する一覧表

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Ⅲ. 研究成果の刊行物・別刷

27

Osteoclast Lineage

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Introduction

Osteoclasts are hematopoietic cells that have bone resorbing activity and participate in bone remodeling and bone marrow formation. Mature functional osteoclasts are large multinuclear cells consisting of multiple osteoclasts fused with each other. Studies of spontaneously arising and gene-targeted osteopetrotic mice have identified molecules essential for osteoclastogenesis. The localization and phylogenetics of osteoclasts are thought to be strictly regulated because these cells are only detected in association with bone.

Since embryonic stem (ES) cells have the potential to differentiate into all cell lineages, it should be possible to derive any cell lineage by appropriate induction of ES cells in culture. *In vitro* studies allow us to manipulate the process of embryonic development and to determine exactly what is happening throughout the entire process of cell differentiation. Moreover, if embryo-like structures could be derived from single ES cells, not only the temporal appearance but also the spatial location of osteoclasts could be studied. Here, we review the biological features of osteoclast development and show our results obtained using ES cell cultures.

Osteoclast Biology

OSTEOCLAST LINEAGE

Osteoclasts, derived from hematopoietic stem cells, participate in bone remodeling and form bone marrow cavities through their bone resorbing activity.[1–4] The precursors share their characteristics with the precursors of monocytic lineage cells, such as macrophages and dendritic cells. Osteoclasts specifically express tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, cathepsin K, and carbonic anhydrase II.[2] They undergo cell fusion with each other, producing large multinucleated cells containing more than 100 nuclei in some cases, and tightly attach to and resorb bone matrices.[1]

Osteoclasts are located on endosteal bone surfaces and the periosteal surface beneath the periosteum, and few are observed in locations without bone.[1,3] A lack of functional osteoclasts results in osteopetrosis, also called "marble bone disease," in which bone marrow cavities are reduced and tooth eruption does not occur.

MOLECULES ESSENTIAL FOR OSTEOCLAST DEVELOPMENT

Analyses of osteopetrotic mice have allowed the identification of molecules essential for generation of the osteoclast lineage (Table 27–1). Especially, two hematopoietic cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL, also named OPGL, ODF, and TRANCE),[5,6] play critical roles in osteoclast development. Mice with mutations in the gene encoding M-CSF, namely $Csf1^{op}/Csf1^{op}$ mice,[7] or in the gene encoding its receptor, Fms, namely *Csf1r*-KO (gene-disrupted) mice,[8] carry severe osteopetrosis. The M-CSF signaling may function in cell survival, because $Csf1^{op}/Csf1^{op}$ mice carrying a *Bcl2* transgene (Tg) are cured of osteopetrosis.[9]

Mice with gene disruption of RANKL *(Tnfsf11)*, classified in the tumor necrosis factor (TNF) superfamily, and its receptor, RANK *(Tnfrsf11a)*, show identical osteopetrotic phenotypes.[10,11] Tg mice overexpressing a decoy receptor for RANKL, osteoprotegerin (OPG, also named OCIF; *Tnfrsf11b*) also harbor osteopetrosis.[12–14]

Molecules acting downstream of RANKL/RANK signaling are known to include TRAF6, NF-κB, mitogen-activated protein kinase (MAPK), and Fos, Fra-1, and Fra-2 (Table 27–1). *In vitro* analysis showed that the addition of inhibitors for p38 and Erk in the MAPK pathway suppresses osteoclastogenesis. Moreover, PU.1-null and dominant-negative MITF mutant *($Mitf^{mi}$)* mice carry osteopetrosis. Anti-E-cadherin antibody inhibits cell fusion of osteoclasts. After cell fusion, bone resorption requires Src function.[15] Recently it was reported that lack of atypical protein kinase C (PKC) scaffold protein (Sqstm1/p62) and nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NF-Atc1/NFAT2) resulted in osteoporosis.[16,17]

ENVIRONMENT FOR OSTEOCLAST DEVELOPMENT

Fms and RANK are expressed simultaneously on osteoclast precursor cells at particular differentiation stages. Arai *et al.*[18] showed that osteoclast precursors in bone marrow develop according to the following sequence: Kit^+ Fms^- $Mac\text{-}1^{dull}$ multipotent cells express Fms, and subsequently they lose Kit expression and become Fms^+ $RANK^+$ $Mac\text{-}1^+$ precursors. M-CSF, RANKL, and OPG are produced by osteoblasts

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TABLE 27–1
Osteopetrotic Mice

Mutated Genes	Products	Mice	Affected Cells
Csf1	M-CSF: macrophage colony-stimulating factor	*op*	Stromal cells
Csf1r	M-CSF receptor–Fms	KO	Osteoclasts
Tnfsf11	RANKL: receptor activator of NF-κB ligand	KO	Stromal cells
Tnfrsf11a	RANK: receptor activator of NF-κB	KO	Osteoclasts
Tnfrsf11b	OPG: osteoprotegerin	TG	Stromal cells
Traf6	TRAF6: TNF receptor-associated factor 6	KO	Osteoclasts
Fos	Fos	KO	Osteoclasts
Fosl1	Fra-1: Fos-like antigen 1	KO	Osteoclasts
Fosl2	Fra-2: Fos-like antigen 2	KO	Osteoclasts
Src	Src	KO	Osteoclasts
Sfpi1	PU.1: SFFV proviral integration 1	KO	Osteoclasts
Mitf	MITF: microphthalmia-associated transcription factor	*mi*	Osteoclasts
Nfkb1, Nfkb2	NF-κB p50, p52	KO	Osteoclasts
Atp6i	H^+ transporting (vacuolar proton pump) member I	*oc*	Stromal cells?
Ostm1	osteopetrosis associated transmembrane protein 1	*gl*	Osteoclasts
Acp5	TRAP: tartrate-resistant acid phosphatase	KO	Osteoclasts
Lifr	LIFR: leukemia inhibitory factor receptor	KO	Osteoclasts
Sqstm1	sequestosome 1, atypical PKC scaffold protein (p62)	KO	Osteoclasts
Nfatc1	NF-ATc1: nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	KO	Osteoclasts

KO, gene-disrupted; TG, overexpressed.

and cloned stromal cells.[2,15] M-CSF is constitutively expressed, but RANKL and OPG expression is regulated by ligands for nuclear factors, parathyroid hormones (PTH and PTHrP), prostaglandins, interleukins, and cytokines.[2–4] Osteoblasts or stromal cells regulate osteoclastogenesis positively and negatively.

The phenotypes of two types of boneless KO mice, namely, runt-related transcription factor 2 (*Runx2*, also named *Cbfal*) and osterix *(Osx)*-gene KO mice, have yielded a key insight into osteoclast development. The transcription factor Runx2 regulates *Osx* expression. Therefore, *Runx2*$^{(-/-)}$ mice lack *Osx*-expressing cells, but *Osx*$^{(-/-)}$ mice contain *Runx2*-expressing cells. *Runx2*$^{(-/-)}$ but not *Osx*$^{(-/-)}$ mice lack osteoclasts *in vivo*,[19,20] whereas osteoclast precursors are present in *Runx2*$^{(-/-)}$ embryos. *In vivo* osteoclast development requires bone tissues, suggesting that the "bone" for osteoclasts is the cells expressing the *Runx2* gene.

Recently, it was reported that T-cells also produce RANKL; for instance, the deterioration of rheumatoid arthritis caused by viral and bacterial infection is related to the production of RANKL by activated T-cells in the joint.[21] Myeloma cells induce RANKL expression in bone marrow stromal cells, and direct RANKL expression by myeloma cells may contribute to enhanced osteoclastogenesis in the bone microenvironment in myeloma bone disease. Furthermore, myeloma cells inhibit the production and induce the degradation of OPG.[22]

BONE MARROW FORMATION

Phylogenetically, the osteoclast lineage first appears in Osteichthyes (bony fishes) among the vertebrates. Zebra fish in which the Fms homologue is deleted lack osteoclasts,[23] indicating that the mechanisms of osteoclastogenesis in bony fishes may be equivalent to those in mammals. Although it is not clear whether RANKL/RANK homologues are present in bony fishes, similar signaling pathways must be present because RANK shares a signaling pathway with Toll-like receptors (TLRs), which are conserved not only in vertebrates but also in invertebrates.[15]

Bone marrow cavities are rudimentary in aquatic animals, such as *Xenopus* (an amphibian) and Trichechiformes (a mammal). This suggests that the presence of osteoclasts is not always linked with bone marrow formation. Intramarrow hematopoiesis first appears in amphibians. The marrow in the land amphibian *Rana* is an active site for lymphohematopoiesis, whereas that in aquatic amphibians such as *Xenopus* is inefficient. The hematopoiesis for the myeloid and erythroid lineages is initiated in rudimentary marrow cavities, and extending the cavities generates B-lymphopoiesis. Bone marrow B-lymphopoiesis is absent in osteopetrotic mice.[24]

To construct the microenvironment for B-lymphopoiesis, sufficient hematopoietic space in the bone marrow may be needed. Therefore, only the bone marrow in land vertebrates produces the B-cell lineage (Table 27–2).

Many menopausal women develop osteoporosis, which occurs because of excessive bone resorption, compared with osteogenesis. Hormonal regulation of B-lymphopoiesis and osteoclastogenesis has been reported. Since estrogen and its derivatives inhibit B-lymphopoiesis, menopausal women have increased B-lymphopoiesis in the bone marrow.[25] Early B-lineage cells are one of the sources of RANKL, and the production of RANKL by B-lineage cells may accelerate bone resorption. Intramarrow B-lymphopoiesis is regulated by the volume of hematopoietic bone marrow cavity, as described previously,[24] and osteoclast differentiation is regulated by B-lineage cell products. Although a most RANKL and M-CSF may be supplied from osteoblasts or stromal cells, the possible relationship between the B- and osteoclast lineages is noteworthy.

TISSUES IN WHICH OSTEOCLAST PRECURSORS ARE PRESENT

Mature osteoclasts, multinucleated TRAP+ cells that resorb bone matrices, are only observed in bone tissues *in vivo.* However, cells that have the potential to differentiate into mature osteoclasts are widely distributed throughout the body, including in the bone marrow, spleen, liver, lung, peritoneal cavity, and peripheral blood.[2,3] During embryonic development, yolk sacs, the aorta–gonad–mesonephros region, and fetal livers contain these precursor cells,[26] and mature TRAP+ osteoclasts are already observed on embryonic day (E) 14. As there are M-CSF and RANKL-producing cells other than osteoblasts, inhibitory molecules such as OPG may regulate osteoclastogenesis *in vivo.* Disruption of the OPG *(Tnfrsf11b)* gene results in severe osteoporosis and atherosclerosis caused by a significant increase of osteoclasts; however, osteoclasts are only present in the bone tissues,[27] suggesting that other regulatory mechanisms must function *in vivo.* We have observed that the development of osteoclast precursors in the bone marrow and spleen, but not in the peritoneal cavity, is induced by TNF-α and M-CSF in the absence of RANKL. The responsiveness to TLR-ligands such as lipopolysaccharide is also different.[28] Thus, the osteoclast precursors maintained in each tissue may not be identical.

Osteoclastogenesis from ES Cells

STEP CULTURES FOR OSTEOCLAST INDUCTION

We established a culture system for the induction of the osteoclast lineage from undifferentiated mouse ES cells. Our step culture system is based on Nakano's coculture system[29] with cloned stromal cells (OP9 cells), as shown in Fig. 27–1. ES cells differentiate efficiently into mesodermal cells and eventually into hematopoietic cells on OP9 stromal cells. The culture medium consists of only a basic medium and fetal bovine serum (FBS). No additional growth factors or cytokines are needed. In this culture system, small clusters of immature hematopoietic cells are observable after 1 week of culture. Mature hematopoietic cells are generated after 10 days of differentiation. Small numbers of colony-forming cells in semisolid media are observed as early as day 5, but at that point, most colonies are of the erythroid and macrophage lineage. In contrast, greater numbers and more types of colonies, including granulocyte, granulocyte and macrophage, and mixed colonies, can be seen on day 10.

By replating the cultured ES cells onto ST2 stromal cells and culturing them in the presence of 1α, 25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] and dexamethasone (Dex) for 6 days, osteoclasts can be generated.[30–32] Interestingly, osteoclast progenitors are present on day 5. This means that the appearance of osteoclast progenitors, like primitive erythrocytes, may precede that of multipotent progenitors. On day 10, more osteoclast progenitors are present. A limiting dilution assay demonstrated that one out of six cells on day 10 are osteoclast

TABLE 27–2
Phylogenetic Comparison of the Presence of Osteoclasts and B-Lymphopoiesis in Bone Marrow

Animals	Mϕ	OC	BM	Intramarrow Hematopoiesis	Intramarrow B-lymphopoiesis
Cartilaginous fishes	Present	Absent	None	Absent	Absent
Bony fishes	Present	Present	None	Absent	Absent
Aquatic amphibians	Present	Present	Rudimentary	Present	Absent
Land amphibians	Present	Present	Present	Present	Present
Land mammals	Present	Present	Present	Present	Present
Osteopetrotic mice	Present	Absent	Rudimentary	Present	Absent
Runx2$^{(-/-)}$ mice	ND	Absent	None	Absent	Absent
Osx$^{(-/-)}$ mice	ND	Present	None	Absent	Absent
Aquatic mammals	Present	Present	Rudimentary	Present	Absent

Mϕ, macrophages; OC, osteoclasts; BM, bone marrow; and ND, not determined.

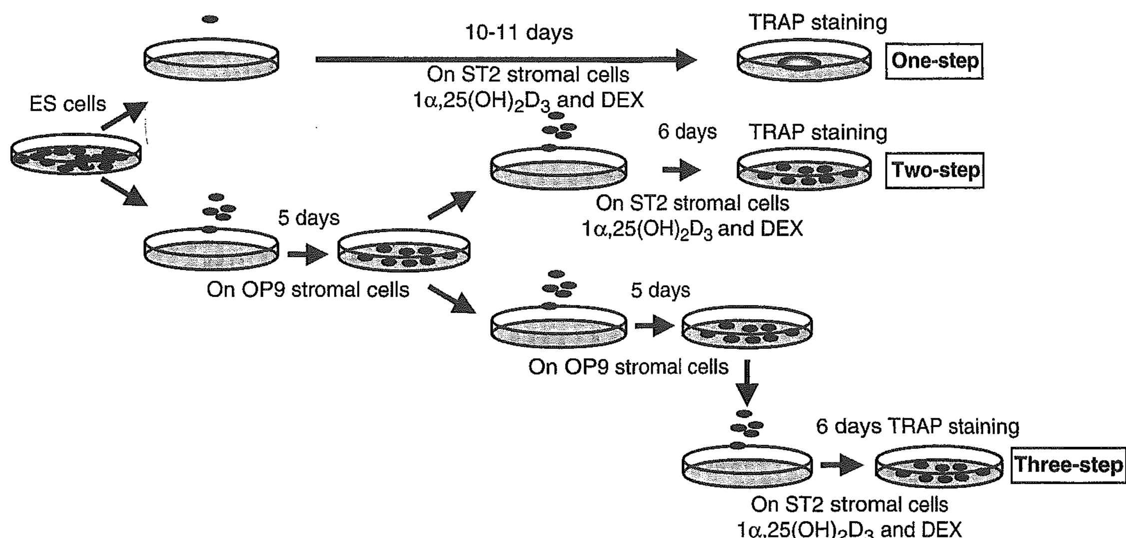

Figure 27–1. ES cell culture system for osteoclastogenesis.

progenitors.[33] It has been reported that during embryogenesis, osteoclast progenitors appear earlier in the yolk sac than in the embryo proper.[26] Our system might reflect the emergence of these osteoclast progenitors at these different stages and different locations.

ONE-STEP CULTURE FOR OSTEOCLAST DEVELOPMENT

The simplest way to induce ES cells to differentiate into osteoclasts is just by putting them in a culture containing $1\alpha,25(OH)_2D_3$ and Dex with ST2 stromal cells. We refer to this culture as a one-step culture (Fig. 27–1). Starting from single ES cells, the cells multiply and form a colony. In the colony, TRAP+ cells are observed on day 8, and on the 10th–11th day of culture, mature functional multinucleated osteoclasts that resorb bone are generated.[34] M-CSF and RANKL may be supplied by ST2 stromal cells. The osteoclastogenesis from ES cells is completely inhibited by continuous addition of OPG, or a monoclonal anti-Fms antagonistic antibody.[33] The requirement for M-CSF precedes that of RANKL, and this order of requirements is identical to that observed for early hematopoietic cells in the bone marrow.[2,15]

Interestingly, the location of mature osteoclasts in colonies is highly specific[30] (Fig. 27–2). Osteoclasts form a circle at the periphery of colonies in the one-step culture. The addition of recombinant soluble M-CSF and RANKL change the site from the periphery to the center of colonies.[34] After mature osteoclasts have been generated in cultures, if the addition of these factors is terminated, osteoclasts become located at the periphery of colonies again. These results appear to indicate that M-CSF and RANKL control the proliferation and differentiation of osteoclast precursors and that the concentrations of M-CSF and RANKL regulate the location of osteoclasts. The influence of the addition of M-CSF and RANKL on the time of the appearance of the osteoclast lineage was also observed; however, we did not find any effect.

OSTEOCLASTOGENESIS FROM ES CELLS THAT LACK HEMATOPOIETIC TRANSCRIPTION FACTORS

Results obtained with an bloodless *Tal1/Scl*-KO ES cell line showed that the Tal1 transcription factor is essential for osteoclastogenesis. However, *Gata1*$^{(-/Y)}$ and *Fog*$^{(-/-)}$ ES cells, which show abnormalities of erythroid, megakaryocyte, and mast cell development, produce normal numbers of mature osteoclasts.[33]

Interestingly, *Gata2*$^{(-/-)}$ ES cells give rise to reduced osteoclast development. The affected stage is a relatively early phase of hematopoiesis. ES cells are induced to differentiate into hematopoietic cells on OP9 stromal cells. On the fifth day, early hematopoietic cells are generated in cultures. These hematopoietic cells contain osteoclast precursors at a frequency of approximately 1/200 in wild-type and 1/3,000 in *Gata2*$^{(-/-)}$ ES cells, respectively. The frequency of osteoclast precursors from *Gata2*$^{(-/-)}$ ES cells is thus significantly reduced.[33] Single precursors from normal and *Gata2*$^{(-/-)}$ ES cells grow and differentiate into comparable numbers of osteoclasts on ST2 stromal cells. These cells are harvested on day 5 and further cultured on OP9 cells for five days. The frequency of

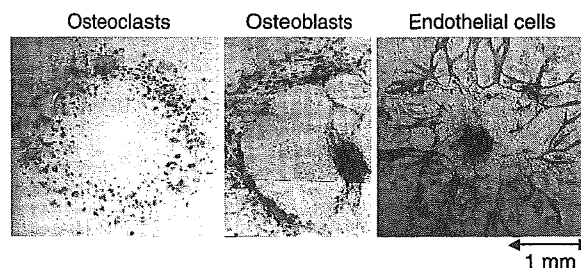

Figure 27–2. *Osteoclasts, osteoblasts, and endothelial cells in ES cell colonies.* Staining of TRAP for osteoclasts (left), alkaline phosphatase (ALP) for osteoblasts (center), and staining with anti-CD31 antibody for endothelial cells (right) were performed on days 10–11 in the one-step culture. (See color plate 4.)

osteoclast precursors the 10th day is a 20-fold increased from that on the 5th day, but the frequency of *Gata2*$^{(-/-)}$ osteoclast precursors is still one-seventh of that of wild-type precursors.[33] These results suggest that the generation of osteoclast precursors from *Gata2*$^{(-/-)}$ ES cells is mainly affected at an early hematogenetic stage within 5 days of the initiation of cultures, and after that, a lack of the *Gata2* gene does not influence osteoclastogenesis. During the first five days after the initiation of cultures, the addition of anti-Fms antibody or OPG does not affect the generation of osteoclast precursors. Surprisingly, the numbers of colony-forming cells elicited by M-CSF (CFU-M) are comparable in cultures of *Gata2*$^{(-/-)}$ and wild-type ES cells, suggesting that osteoclast precursors and CFU-M may not be identical,[33] although CFU-M has the potential to differentiate into osteoclasts in culture.[35]

KO mutations of all of these genes (*Tal1/Scl, Gata1, Fog,* and *Gata2*) are early embryonic lethal, and there have been no reports on osteoclast development in these mutants *in vivo.* The ES cell culture system enables us to assess the function of such genes in osteoclast development (Fig. 27–3).

LOCATIONS OF OSTEOCLAST, OSTEOBLAST, AND ENDOTHELIAL CELL LINEAGES IN ES CELL COLONIES

In the one-step culture, we can observe a wider range of cell lineages in a dish compared with OP9 cultures. In addition to hematopoietic lineages, at least endothelial cells, osteoblasts, myocardial cells, melanocytes, and pigmented epithelial cells are observed. Hematopoietic cells and endothelial cells share progenitor cells, called hemangioblasts, or endothelial cells are progenitor cells for hematopoietic cells. During embryogenesis, hematopoiesis and vasculogenesis are first observed in extraembryonic yolk sacs on E7.0–7.5. In the one-step culture, Kit^{high}, β2-integrin–expressing hematopoietic cells, and CD31- and Flk1-expressing endothelial cells are first observed on day 4. Mature osteoclasts are observed on E14 in embryos and on days 10–11 in culture.[34] The time required for the derivation of ES cells from blastocysts (E3.5–4.0) may account for this difference of timing. Therefore, the program of cell differentiation in this culture system is likely to occur with precisely the same timing as embryogenesis *in vivo.*

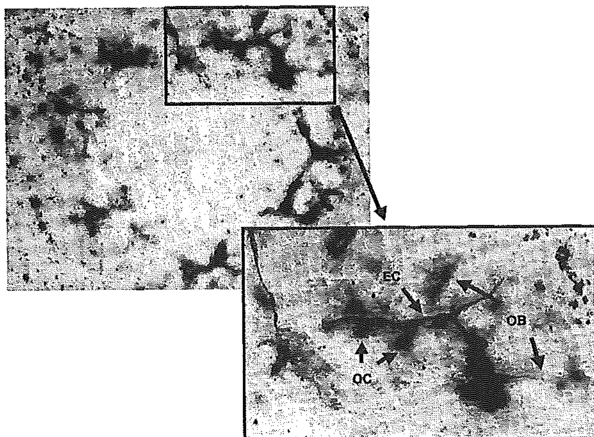

Figure 27–3. Triple staining of TRAP, ALP, and anti-CD31 of an ES cell colony on day 11 in the one-step culture on ST2 stromal cells (OC, osteoclasts; OB, putative osteoblasts; and EC, endothelial cells). (See color plate 5.)

Endothelial cells have a striking localization pattern in colonies[34] (Fig. 27–2). Vasculogenesis in *Tal1*-KO ES colonies, which lack osteoclasts, occurs normally and forms a similar pattern to that in wild-type colonies. The addition of M-CSF and RANKL changes the site of osteoclasts in the colonies but not that of endothelial cells.[36] This may mean that the pattern of endothelial cell generation is determined by the cells alone.

Bone marrow formation involves the participation of three lineages of cells. Osteoblasts build the bone, endothelial cells invade the bone, and osteoclasts resorb the bone and make the bone marrow cavity. Alkaline phosphatase-positive (ALP+) osteoblast-like cells appear on day 8 and are present at relatively inner sites of the colonies compared to osteoclasts. These two lineages of cells are closely associated and located as concentric circles.[36,37] ALP+ cells are derived from ES cells, not from the underlying ST2 stromal cells. Triple staining for putative osteoblast, osteoclast, and endothelial cell lineages is shown in Fig. 27–3.[37] Endothelial cells, osteoblasts, osteoclasts, and ST2 bone marrow stromal cells are located in an orderly pattern from the center to the outside of colonies. The locations seem to correspond to those of the bone marrow turned inside out. Even *in vitro,* there are still some rules regulating the localization of the cell lineages in colonies in the dish. These observations suggest that each cell lineage generated in the colonies has a preferred position, interacts with other lineages, and is subject to regulation of its growth and differentiation, temporally and spatially.

OSTEOCLASTOGENESIS FROM ES CELLS WITHOUT SUPPORTING STROMAL CELLS

As described in the previous section, coculture systems of single ES cells with ST2, OP9, or both stromal cells work well for the production of mature osteoclasts. However, if we want to know how undifferentiated ES cells regulate themselves and construct a tissue-like structure *in vitro,* the influence of precommitted cells in cultures has to be excluded. To induce osteoclasts under stromal cell-free conditions, at least some cells derived from the ES cells must differentiate into osteoclast precursors, and some must differentiate into supportive cells. We found that ascorbic acid is the critical reagent for osteoclastogenesis from ES cells without cloned stromal cells. The effects of ascorbic acid not only on osteoblast development (as reported) but also directed to osteoclasts may be important.[31,38] Although we have not generated osteoclasts from single ES cells yet, the development of a single ES cell culture system without stromal cells will allow us to study how organogenesis proceeds *in vitro.*

Culture Methods

ONE-STEP CULTURE ON ST2 STROMAL LAYER[30,31]

1. Prepare confluent ST2 feeder layer in 24-well plates (Note a1).
2. Grow ES cells to a subconfluent state and harvest them.

3. Seed ES cells at the appropriate cell density (Note a2). Supplement with 10^{-8} M $1,25(OH)_2D_3$ and 10^{-7} M Dex (final concentration).
4. Change the culture medium every two or three days.

Medium used: α-minimum essential medium (α-MEM: Gibco-BRL) supplemented with 10% FBS (see Note a3), 50 U/ml streptomycin, and 50 μg/ml penicillin.

Notes:

a1. ST2 cells are maintained in RPMI-1640 supplemented with 5% FBS, 50 μM 2-ME, 50 U/ml streptomycin, and 50 μg/ml penicillin.

a2. Seed ES cells so that about 20 colonies are generated per well. Plating efficiency varies according to the lot of serum.

a3. The appropriate lot of FBS must be selected.

MULTISTEP CULTURE ON OP9 STROMAL LAYER

1. Prepare confluent OP9 feeder layer in 6-well plates (Note b1).
2. Grow ES cells to a subconfluent state and harvest them.
3. Seed 10^4 ES cells per well.
4. On day 2 or 3 of differentiation, replace half of the medium with fresh medium.
5. On day 5 of differentiation, colonies that have a differentiated appearance will be observed. After washing the cultures with PBS, trypsinize them with 0.25% trypsin/0.5 mM EDTA for five minutes at 37°C. Dissociate the cell clump by pipetting up and down vigorously. After centrifugation, count ES-derived cells. Do not count OP9 cells. They are large and easily distinguished from ES cell-derived cells. About $1–2 \times 10^6$ cells are obtained per well. For two-step cultures, refer to the "Induction of Differentiation to Osteoclasts in Multistep Cultures" section. For three-step cultures, follow the steps here.
6. Seed 10^5 ES cells per well of 6-well plates containing freshly prepared OP9 layers.
7. On day 7 or 8 of differentiation, change half of the medium gently.
8. On day 10 of differentiation, hematopoietic clusters or colonies will have formed on the OP9 layers. Harvest the cultured cells by pipetting up and down. Let them stand for 4–5 minutes to precipitate the debris of OP9 stromal cells. Transfer the supernatant into a fresh tube. After centrifugation, count ES-derived cells. About 10^5 hematopoietic cells will be obtained per well. For three-step cultures, refer to the "Induction of Differentiation to Osteoclasts in Multistep Cultures" section. If you want to simultaneously analyze the other hematopoietic lineages, plate the cell suspension again onto fresh OP9 at 10^5 cells per well in 6-well plates.

Medium used: α-MEM (Gibco BRL) supplemented with 20% FCS (Note b2), 50 U/ml streptomycin, and 50 μg/ml penicillin.

Notes:

b1. OP9 are maintained in α-MEM supplemented with 20% FBS, 50 U/ml streptomycin, and 50 μg/ml penicillin.

b2. The appropriate lot of FBS must be selected.

INDUCTION OF DIFFERENTIATION TO OSTEOCLASTS IN MULTISTEP CULTURES

1. Prepare confluent ST2 feeder layers in 24-well plates (Note a1).
2. For two-step cultures (from step 5 in the "Multistep Culture on OP9 Stromal Layer" section), seed $0.5–1\times 10^4$ cells per well. For three-step cultures (from step 12 in the "Multistep Culture on OP9 Stromal Layer" section), seed 10^3 cells per well.
3. Culture cells for six days in α-MEM supplemented with 10% FBS, 50 U/ml streptomycin, and 50 μg/ml penicillin, 10^{-8} M $1,25(OH)_2D_3$ and 10^{-7} M Dex.
4. Change the culture medium every two or three days.

TRAP STAINING

1. Aspirate the culture medium.
2. Add 1 ml of 10% formalin (3.7% formaldehyde) in PBS (v/v) to each well of the plates, and fix them for 10 minutes at room temperature.
3. After washing with PBS, cover with 0.5 ml of ethanol/acetone (50:50 v/v) for exactly 1 minute at room temperature. After the treatment, immediately fill each well with PBS, aspirate the solution, and wash once more with PBS.
4. After the aspiration of PBS, cover the fixed cells with 0.25 ml of TRAP staining solution, and incubate for 10 minutes at room temperature. After red color develops, wash the plates well with water. Insufficient washing will generate high background staining.

TRAP staining solution: Acetate buffer (pH 5.0) containing 50 mM sodium acetate, 25 mg/ml naphthol AS-MX phosphate (Sigma) in a dark glass bottle at 4°C. Just before use, dissolve fast red violet LB salt (Sigma) in the volume you need at the final concentration of 0.5 mg/ml.

Summary

Osteoclasts are important cells for bone cell biology, as osteocytes and chondrocytes. It is clear phylogenetically at which point this cell lineage arises. Moreover, for assessing the hematopoietic potential of ES cells or very early embryos, the osteoclast lineage is convenient. Osteoclasts are specialized, large multinuclear cells. TRAP is a very stable enzyme, and staining for it is easy and specific. Since a majority of blood cells are nonadherent cells, it is hard to define the hematopoietic sites in culture. Osteoclasts are known to be among the most tightly adherent cells to dishes, making it possible to examine the spatial location of the cells in cultures. Macrophages are also adherent cells; however, macrophages keep the potential to differentiate into osteoclasts and dendritic

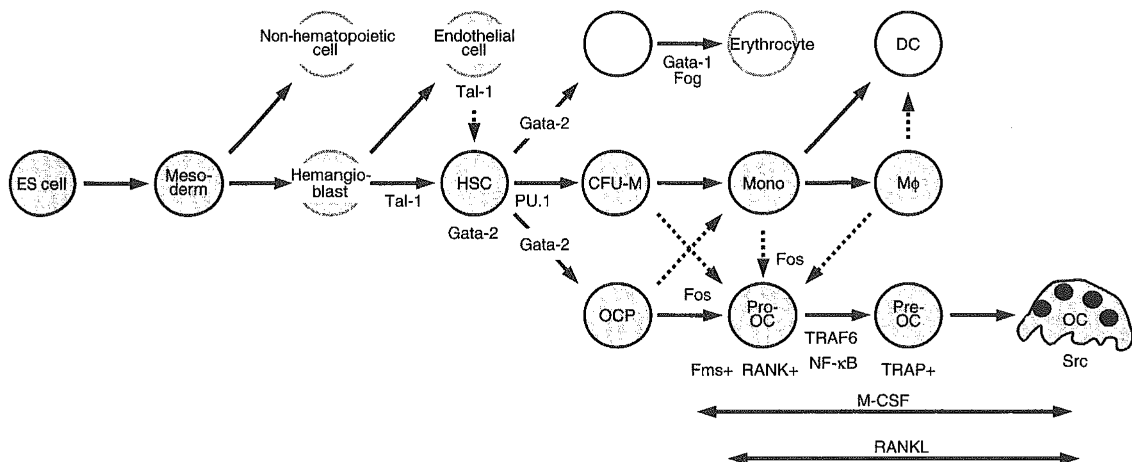

Figure 27–4. Hypothetical scheme of osteoclast development from undifferentiated ES cells (HSC, hematopoietic stem cell; Mono, monocyte; DC, dendritic cell; Mϕ, macrophage; OCP, osteoclast precursor; and OC, osteoclast).

cells, meaning that some "macrophages" may be precursor cells and change their characteristics.

Molecules essential for embryonic development, including molecules involved in Notch and Wnt signaling, regulate osteoclastogenesis directly in precursors and through supporting microenvironments *in vitro*.[39,40] A recent report showed that TNF-α, and even LPS in some conditions, can substitute for the function of RANKL in culture.[41,42] However, the authors that produced *Tnfsf11*-KO mice emphasized that RANKL/RANK is essential for osteoclastogenesis *in vivo*.[15] Open questions about the differences between *in vivo* and *in vitro* osteoclast biology remain (Fig. 27–4).

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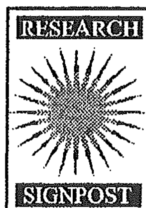

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3 Migration of dendritic cells determines divergent immune responses

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Abstract

Dendritic cells (DCs) are the most potent regulator of the immune system. DCs are heterogeneous with respect to their origin, distribution and functions. They arise from hematopoietic precursor cells and differentiate into several DC subsets. They are distributed in both the parenchyma and epithelia of many organs, and keep watch for harmful invading antigens (Ags). They direct distinct functions in the immune system: they activate Ag-specific adaptive immunity, link Ag-nonspecific innate immunity and

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Ag-specific adaptive immunity, and induce "immune tolerance". Although how DCs direct such distinct functions is still not clear, differences in their subsets, distribution or migration process to secondary lymphoid organs, where DCs present captured Ags to naive T cells, might be related to specifying their functions.

DCs act as immune stimulators when they capture invaded Ags and migrate to secondary lymphoid organs in the active state. On the other hand, it has been suggested that when DCs migrate to secondary lymphoid organs in "the steady state", they seem to act as tolerance-inducing cells. Using hyperpigmented transgenic mice, we have observed steady-state trafficking of skin Ags transported by Langerhans cells (LCs) (skin- resident DCs), and further found the distinct migration of LCs in the steady and active state. Our findings indicated that abrogation of steady-state trafficking seemed to cause autoimmunity.

In this review we discuss the relation between DC migration and selection of the immune response - immunogenic or tolerogenic.

Introduction

Dendritic cells (DCs) are known as hematopoietic-lineage, potent antigen presenting cells (APCs) that capture foreign antigens (Ags) such as bacteria and viruses. DCs process and present Ags to naive T cells, then activate immune responses against Ags (1).

Although DCs were long considered to activate immune responses only against harmful foreign Ags, recent studies have suggested that DCs also regulate "immune tolerance", the suppression system for harmful immune responses against self Ags/ tissues (2-4). Ag-presentation by DCs is the first step in the activation of immune responses, and Ag-captured DCs have to migrate to secondary lymphoid organs, i.e., regional lymph nodes (LNs) or the spleen. Such migration may occur when foreign Ags enter tissues and act as stimuli; this is referred to as the "active-state" migration of DCs. On the other hand, the migration of DCs also occurs constitutively without stimuli, a phenomenon called the "steady-state" migration of DCs (2-8). Although it is still not clear how DCs regulate both immune responses to foreign Ags and immune tolerance to self-Ags, it has been suggested that the steady-state migration of DCs is deeply related to the regulation of immune tolerance. Thus the patterns of DC migration - active- versus steady-state migration - seem to hold the key to whether an immune response or immune tolerance occurs.

Recently, we clearly showed the presence of steady-state migration of skin DCs using hyperpigmented transgenic mice (Tg) (6,9), and demonstrated that the steady- and active-state migration of Langerhans cells (LCs: DCs in the epidermis) from the skin is regulated distinctly (10). Based on our findings we