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Stochastic differentiation into an osteoclast lineage from cloned macrophage-like cells

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ABSTRACT

Differentiation into osteoclasts is induced by a macrophage colony-stimulating factor and receptor activator of nuclear-factor κB ligand. The macrophage-like cell line, C7 has the potential to differentiate into osteoclasts when it is cultured with both factors for 6 days. Although C7 is an established cell line, the frequency of differentiation into this lineage was less than 10%, and the ratio was maintained at a constant level, even after repeated cloning. In this study, to increase the differentiation of C7 cells to osteoclasts, C7 derivative treatments with several activators and/or inhibitors were performed for 3 days prior to setting osteoclast induction analysis; however, a reagent to significantly up-regulate the frequency of differentiation was not found. Only extended cultures for osteoclastogenesis exponentially increased the frequency of osteoclast precursors. It is likely that C7 cell differentiation into committed osteoclast precursors is on 'autopilot' rather than requiring specific signals to drive this process.

1. Introduction

Osteoclast precursors (OCPs) are thought to be classified in a monocytic lineage, and differentiate into osteoclasts in the presence of a macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [1–6]. Almost all cells in the monocyte fraction from human peripheral blood cells were reported to differentiate into osteoclasts [7]. Mouse bone marrow macrophages (BMMs), induced by M-CSF, and RAW264.7, a macrophage-like cell line, are also known to give rise to osteoclasts at a high ratio [8].

We previously established the macrophage-like cell line, C7 and its derivative C7-TY [9]. These give rise to osteoclast-like tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) that resorb bones when they are cultured with M-CSF and RANKL [6,10,11]. However, the ratio of their differentiation into osteoclast-like cells is sporadic at less than one-tenth and is relatively constant [9]. Differentiation into osteoclasts seems to occur in only a part of C7 and C7-TY cells. It is not clear whether a part of these cell lines change the phenotype or if they still consist of a mixture of different cell phenotypes.

A surprising consistency was observed in two other cell lines that were independently established. One was a BDM-1 cell line from a BMM cultured with M-CSF [12], and another was a SV40 T antigen-transgenic mouse-derived monocyte line [13]. They were reported to give rise to osteoclast-like cells in a ratio at 5–9% and 5–8%, respectively [12,13]. What mechanism(s) regulates the constant rate of osteoclastogenesis from C7 cells?

In this study, to define stimuli-controlling differentiation and/or commitment into an osteoclast lineage, recloned C7-TY-8 cells were pre-treated with a variety of activators or inhibitors for 3 days and induced osteoclastogenesis by M-CSF and RANKL for 6 days. Some stimuli increased the frequency of differentiation, but the range was partial up to 0.136. Only the extension of the culture term for osteoclastogenesis could increase the frequency. Although it still remains a possibility that unknown stimuli we have not tested are essential, autonomous regulation may be involved in the commitment of C7 cells into an osteoclast lineage.

2. Materials and methods

2.1. Cells

Trp53-null BM cell-derived cloned macrophage-like cells, C7-TY cells [9–11] were maintained in α-MEM (Life Technologies, Grand

Abbreviations: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D3; BMM, bone marrow macrophage; mAb, monoclonal antibody; MNC, multinucleated cell; OCP, osteoclast precursor; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase.

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Island, NY) supplemented with 10% fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS), recombinant human M-CSF (a kind gift from Dr. Masayuki Takahashi, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan), and antibiotics (penicillin and streptomycin; Meiji Chemical Co. Ltd., Tokyo, Japan).

A C7-TY cell had been cloned from original C7 cells by maintaining it in 50 ng/ml recombinant human M-CSF after repeated passages [10,11]. To be clear of its clonality, C7-TY cells were re-cloned in a 700-well hybridoma dish (a well size: 2 mm × 2 mm, Greiner Bio-One, Co. Ltd., Kremsmünster, Austria) (Supplementary Fig. S1A). One of the 39 re-cloned cells, C7-TY-8, showed a similar frequency in TRAP$^+$-wells (0.0959) with parental C7-TY cells (0.100) (Supplementary Fig. S1B). We mainly used this cell line following experiments, unless otherwise indicated. Cells were harvested with 0.25% trypsin (Life Technologies)/1 mM EDTA/PBS (Ca^{2+}, Mg^{2+}-free) for 15 min at room temperature.

RAW264.7, a BALB/c Abelson murine leukemia virus transformed macrophage-like cell line [14], was a gift from Dr. Shumpei Niida (National Center for Geriatrics and Gerontology, Aichi, Japan) and was maintained in 10% FBS/α-MEM/antibiotics.

BMMs were induced by culturing freshly prepared BM cells ($7–8 \times 10^6$) in 10 ml 10% FBS/α-MEM supplemented with 50 ng/ml M-CSF for 3 days in 10-cm suspension culture dishes (Corning Costar, Corning, NY) [10]. Adherent cells were harvested with 0.25% trypsin/1 mM EDTA/PBS after washing with PBS as a BMM.

2.2. Differentiation into osteoclasts

As shown in Supplementary Fig. S2, 50 ng/ml M-CSF and 50 ng/ml recombinant human soluble RANKL (PeproTech EC Ltd., London, UK) were generally used to induce osteoclastogenesis. Osteoclastogenesis from RAW264.7 cells was performed without M-CSF. In some experiments, a fusion protein comprising GST and the extracellular domain of human RANKL (amino acid residues 140–317; GST-RANKL) from Oriental Yeast (Tokyo, Japan) was used as RANKL [5,6]. Cultured cells were fixed with 10% formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS for 10 min and with ethanol–acetone (50:50, v/v) (Wako) for 1 min at room temperature, and were incubated in acetate buffer (pH 5.0) (Sigma–Aldrich, St. Louis, MO) containing naphthol AS-MX phosphate (Sigma–Aldrich) as a substrate and fast red violet LB salt (Sigma–Aldrich) as a stain in the presence of 50 mM sodium tartrate (Wako) [15] (See Supplementary Fig. S1).

2.3. Frequency analysis of OCPs

C7-TY, and its derivatives, were cultured in 700-well hybridoma dishes (2 mm × 2 mm square per well) at 700 cells/dish with 8 ml 10% FBS/α-MEM supplemented with 50 ng/ml M-CSF and RANKL. Half of the medium (4 ml) was replaced with fresh medium containing double doses of M-CSF and RANKL every 3 days.

Alternative frequency analysis of OCPs was performed by a limiting dilution assay [16]. One or 5 harvested cells were inoculated per well in 96-well plates (Corning Costar) containing 0.1 ml medium supplemented with 50 ng/ml M-CSF and RANKL for 6 days. The presence of osteoclast-like cells was determined by TRAP staining.

2.4. Pre-cultures for inducing OCPs

C7-TY-8 cells in 10-cm dishes were passed to new dishes on a weekly basis. To assess the frequency of giving rise to TRAP$^+$ cells, 10^4 C7-TY-8 cells were cultured in 12-well plates with or without several reagents in the presence of 50 ng/ml M-CSF. Here, this phase was named "pre-culture" and was performed for 3 days. Previously, we reported C7-TY cells gave rise to TRAP$^+$ cells on the 4th day of culture, but few TRAP$^+$ cells were observed for 3 days with M-CSF and RANKL. On the third day of culture, 10^4 C7-TY-8 cells gave rise to 13 ± 2 TRAP$^+$ cells and 2 ± 2 TRAP$^+$ MNCs only [10]. Therefore, we selected 3-day pre-culturing because it is the longest term without the appearance of TRAP$^+$ cells. Subsequently, harvested 700 C7-TY-8 cells from pre-cultures were seeded in 700-well hybridoma dishes with 50 ng/ml M-CSF and 50 ng/ml RANKL.

2.5. Statistical analysis

Data were presented as means ± S.D. Statistical analysis was carried out by the Student's *t*-test. The level of statistical significance was present at $p < 0.05$.

3. Results

3.1. The frequency of TRAP$^+$ cell-containing wells in C7-TY-8 cell cultures was relatively constant

Re-cloned C7-TY-8 cells cultured with 50 ng/ml M-CSF were repeatedly passed to 10-cm culture dishes, and first, we assessed the frequency of OCPs in C7-TY-8 cells by indicating the numbers of TRAP$^+$ cell-containing wells (hereafter TRAP$^+$-wells). We successively performed 27 time-repeated frequency analyses using 700-well hybridoma dishes (Supplementary Fig. S1A). In the majority of experiments, the frequency in 6-day cultures was lower than 0.1, and the maximal response (0.136) was experienced in the thirteenth experiment (the range of OCP frequencies: 0.00286–0.136) (Fig. 1).

Since growth signal(s) such as M-CSF may be involved in differentiation/commitment into the osteoclast lineage, we compared the frequency of differentiation into TRAP$^+$ cells of C7-TY-8 cells pre-cultured in 12-well plates with or without M-CSF for 3 days. Recovered numbers of cells without M-CSF were significantly lower than those with M-CSF [the ratio of recovered cell numbers cultured without versus with M-CSF was 0.134–0.325; hereafter referred to as 0.134 fold (×)–0.325×].

However, the frequency of TRAP$^+$-wells was not reduced (Table 1, lane 2). It coincided with pre-culturing with AFS98, an M-CSF receptor antagonistic monoclonal antibody (mAb) to block M-CSF activity (Table 1, lane 3). It is unlikely that growth signaling via the M-CSF receptor is critical in the commitment of C7-TY-8 cells into OCPs.

3.2. Pre-culturing C7-TY-8 cells with a variety of reagents did not significantly increase the frequency of OCPs

Why is the ratio of C7-TY-8 cells differentiating into TRAP$^+$ cells low? There are two possible explanations; the insufficiency of

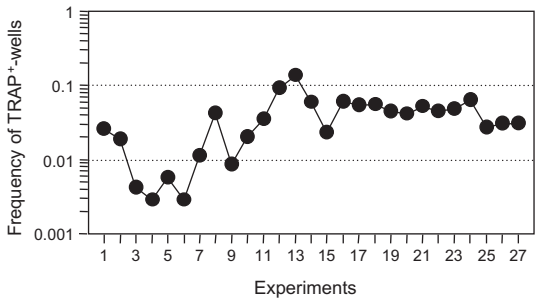

Fig. 1. The frequency of differentiation into TRAP$^+$ cells from C7-TY-8 cell repeated passages. C7-TY-8 cells cultured with 50 ng/ml M-CSF in 10-cm dishes were repeatedly passed and the frequency of TRAP$^+$-wells was assessed. In 27 successively repeated analyses, the frequency was 0.00286–0.136. The maximal response (0.136) was experienced in the thirteenth experiment.

Table 1
Reagents used in this study.

Reagents	Concentration	Controls	Cell recovery	Frequency of TRAP$^+$ cell generation
			Effect (fold with control)	Effect (fold change with control: maximal frequency)
M-CSF	50 ng/ml	Medium only	↑(3.08×–7.44×)	(0.67×–)
Medium only		M-CSF	↓(0.13×–0.33×)	(1.50×)
Anti-Fms mAb	5 μg/ml	+ACK4	↓(0.20×)	(1.17×)
RANKL	25 ng/ml	M-CSF	(1.00×)	↓(0.33×)
OPG	200 ng/ml	M-CSF	0.63×–1.39×	↑(**1.67×–5.52×: max 0.0202**)
GM-CSF	100 U/ml	M-CSF	(1.04×)	↓(0.33×)
Anti-GM-CSF antiserum	0.5% v/v	+rabbit serum	↑(1.91×)	↓(0.61×)
IFN	100 ng/ml	M-CSF	↓(0.44×)	↓0
TGF-β1(R47)	50 ng/ml	M-CSF	→(0.86×)	↓(0.62×)
VEGF164	100 ng/ml	M-CSF	→(1.04×)	(0.83×)
VEGFR1/Fc	100 ng/ml	+B7–1/Fc	→(1.20×)	→(0.93×)
VEGFR2/Fc	100 ng/ml	+B7–1/Fc	→ (1.10×)	→ (1.18×)
Flt-3L	100 ng/ml	M-CSF	→ (1.42×)	→ (0.83×)
Anti-Flt-3 mAb	5 ng/ml	+ACK4	↓(0.50×)	↑(**2.57×: max 0.0558**)
ETRA antagonistic peptide	100 nM	M-CSF	→ (1.33×)	↓(0.62×)
ETRB antagonistic peptide	100 nM	M-CSF	→ (1.26×)	↓(0.65×)
BMP2	10 ng/ml	M-CSF	↓(0.57×)	↑(**2.18×: max 0.128**)
BMP4	10 ng/ml	M-CSF	→ (0.70)	↑(**1.72×: max 0.101**)
BMPRI-A/Fc	100 ng/ml	+B7–1/Fc	→(1.39×)	→(0.72×)
Anti-TNFα mAb	5 μg/ml	+ACK4	→(1.00×)	↓(0.50×)
Sodium thiophosphate	100 μM	M-CSF	→(1.16×–1.24×)	↑(**1.17×–3.81×: max 0.0438**)
Cyclopamine	5 nM–5 μM	+EtOH	→(0.45×–0.92×)	→(**0.73×–1.47×: max 0.118: 0.5 μM**)
Wnt-3a-L cell SN	10–25% v/v	+Neo-L cell SN	→(0.84×–1.20×)	→(0.95×-1.18×)
WIF-1/Fc	100 ng/ml	+B7–1/Fc	→ (0.80×)	→(0.67×)
BIO	5 nM–5 μM	+DMSO	0.13×–1.25×	**0.44×–1.41× (max 0.120: 5 nM)**
Dexamethasone	100 nM	M-CSF	↓(0.25×)	↓(0.17×)
1α,25(OH)$_2$D$_3$	10 nM	M-CSF	→(0.90×)	↑(**1.67×**)
DMSO	0.01% v/v	M-CSF	0.57×–1.29×	→(0.80×–1.29×) (max 0.0528)
Detla1-FL-coated	10–100 μg/ml	+BAP-Flag-coated	→(1.16×–1.21×)	→(0.67×–1.00×)
Jagged1-FL-coated	10–100 μg/ml	+BAP-Flag-coated	→(1.11×–1.21×)	↓0
DAPT	10 nM–100 μM	+DMSO	0.65×–2.32×	**0.60×-2.29× (max 0.195: 1 βM)**
PD098059	20 μM	+DMSO	↓(0.32×)	→(0.67×)
SB203580	20 μM	+DMSO	↓(0.37×)	↓(0.17×)
SP600125	20 μM	+DMSO	↓(0.084×)	↓0
Cyclosporin A	1 μM	+DMSO	→(1.03×)	↓(0.36×)
FK506	0.1 μM	+DMSO	→ (0.86×–1.12×)	↓(0.54×)
Anti-Kit mAb (ACK4)	5 μg/ml	M-CSF	0.72×–1.58×	**0.51×-2.52× (max 0.0275)**
Anti-FcγR mAb	5 μg/ml	+ACK4	→ (1.08×)	↓(0.52×)
B7–1/Fc	100 ng/ml	M-CSF	0.50×–0.96×	**0.96×-2.01× (max 0.118)**
LPS	20 ng/ml	M-CSF	→ (0.87×)	↓0
Anti-RP105 mAb	5 μg/ml	+ACK4	→ (0.88×)	↓(0.21×)
Anti-CD44 mAb	5 μg/ml	+ACK4	→ (0.98×)	↓(0.37×)
Anti-VCAM-1 mAb	5 μg/ml	+ACK4	↑(1.82×)	↓(0.21×)

→: Range between 0.67× and 1.50×, ↑: increased (> 1.50×), ↓: decreased (< 0.67×). Controls shown + mean 50 ng/ml M-CSF plus reagents in control cultures for 6 days.

critical factor(s) or the presence of suppression factor(s) for the commitment of C7-TY-8 cells into OCPs during their maintenance. To assess these possibilities, 3-day inductive pre-cultures with various inhibitors or stimulators of some signaling cascades were performed prior to the final differentiation culture with M-CSF and RANKL for 6 days. The effects of reagents added in pre-cultures were shown in Table 1 and Supplementary Table S1. An appropriate concentration added to pre-cultures for each reagent was decided by referring ours or other previous reports (see Supplementary references in Supplementary Table S1).

The frequency of TRAP$^+$-wells was increased by applying some of these reagents, including sodium thiophosphate (an SHP-1 inhibitor), OPG (a decoy receptor for RANKL), VEGFR2/Fc, A2F10 (an antagonistic anti-Flt-3 monoclonal antibody), 1α,25(OH)$_2$D$_3$, DAPT (a γ-secretase inhibitor in the Notch signaling pathway), BMPs, cyclopamine (a smoothened inhibitor in the hedgehog signaling pathway), and BIO (an inhibitor of GSK-3 in the Wnt signaling pathway) (Table 1). Several antibodies, which are not dependent on their specificities or species, and recombinant-Fc of human IgG1 sometimes increased the frequency.

Since pre-cultures with each reagent could not exceed 0.2 (0.195: M-CSF + DAPT 1 μM) in the frequency of TRAP$^+$-wells, we mixed the reagents (sodium thiophosphate, OPG, VEGFR2/Fc, A2F10, 1α,25(OH)$_2$D$_3$, ACK4 for Fc, and DMSO), pre-cultured C7-TY-8 cells with the mixture for 3 days, and assessed the frequency (Table 1 and Fig. 2A). A mixture of DAPT, BIO, and/or cyclopamine was also used as a stimulator, but significant increases in the frequency were not observed in either pre-culture (Fig. 2). These results indicated that any extracellular stimulation we tested did not dramatically affect the commitment of C7-TY-8 cells into OCPs.

To assess the effect of cell concentration on the frequency of TRAP$^+$ cell generation, a serial area of culture wells and dishes were used for pre-culturing. However, a significant difference was not observed (Supplementary Fig. S3).

3.3. Cell numbers in TRAP$^+$-wells and frequencies of TRAP$^+$-wells were increased as the number of culture days increased

As shown above, we performed experiments to up-regulate the generation of wells containing TRAP$^+$ cells, but the addition of various reagents or alterations in cell density in pre-cultures did not show any striking effects. Although it is still possible that unknown stimuli not tested here are essential, we could not promote the

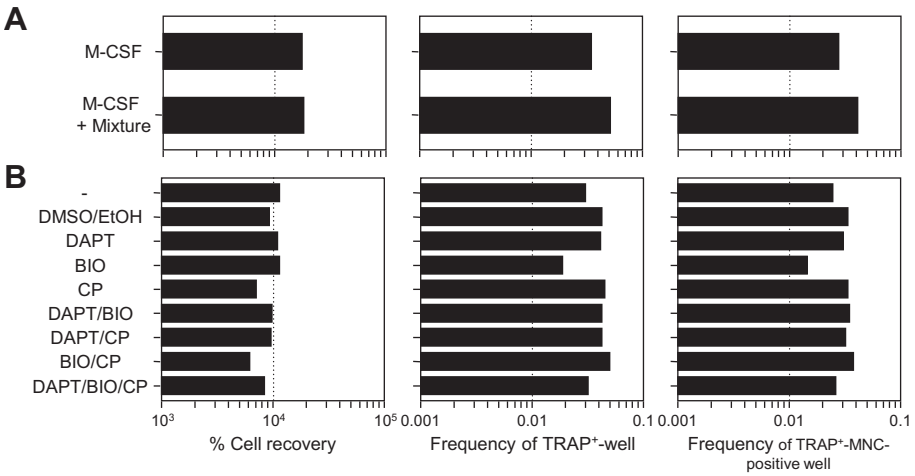

Fig. 2. The effect of pre-culture with a mixture of selected reagents on the frequency of osteoclast precursors. (A) C7-TY-8 cells (10^4/well) were pre-cultured with a mixture consisted of 50 ng/ml M-CSF, 100 μM sodium thiophosphate, 200 ng/ml human OPG, 100 ng/ml mouse VEGFR2/Fc, 5 μg/ml A2F10, 10 nM 1α,25(OH)$_2$D$_3$, 5 μg/ml ACK4 for Fc, and 0.01% v/v DMSO. On day 3, cells were harvested, cell numbers were counted, and % recovery was shown (left). Using 700 harvested cells, the frequency was assessed for 6 days. Frequencies of TRAP$^+$-wells and TRAP$^+$-MNCs-positive wells were shown in center and right graphs, respectively. (B) Pre-cultures with a mixture of 1 μM DAPT, 5 nM BIO, and/or 0.5 μM cyclopamine (CP) were used as a stimulator. DMSO and EtOH were added at 0.01% v/v and 0.1% v/v, respectively.

commitment of C7 cells into OCPs. This stable frequency may account for the presence of a stochastic/probabilistic process under autonomous regulation [17]. If this osteoclastogenesis from C7 cells is regulated stochastically, it must be repeated in each well of the hybridoma dishes. When the number of growing cells reaches that of proper ones, osteoclastogenic C7 cells emerge in the well.

To assess this possibility, we performed extended cultures to increase cell numbers in each well. The final differentiation phase with M-CSF and RANKL in 700-well dishes was extended from 6 to 22 days. Total cell numbers in single TRAP$^+$-wells were also continuously increased with increasing culture days; on day 22, an average of 245 cells were in a well (Fig. 3A). In other experiment observed up to day 30, the number of cells reached 798 ± 264 cells/well. Since the well size of a hybridoma dish is 2 mm × 2 mm squared, the maximum cell number in a confluent well should be 700–1000 cells.

On the 3rd, 6th, 12th, 17th, and 22nd day in hybridoma dishes, the number of TRAP$^+$-wells was counted (Fig. 3B). No TRAP$^+$ cell was observed on day 3 of culture. After 6 days, the number of TRAP$^+$-wells continuously increased with a corresponding increase in culture days (Fig. 3B). The frequency of TRAP$^+$-wells lineally increased, and reached 0.56 and 0.83 on the 17th and 22nd day (299 and 395 wells in 700 wells contained TRAP$^+$ cells), respectively. Those of TRAP$^+$ MNC$^+$-wells also increased to 0.53 and 0.71 on the 17th and 22nd day, respectively. As shown in Supplementary Fig. S4, the plating efficiency of C7-TY-8 cells in hybridoma dishes was 36.9 ± 4.1, meaning that if the number of TRAP$^+$-wells is 258 ($700 \times 0.369 = 258.3$, frequency: $700/700(\ln(700/(700-258))) = 0.460$), almost all cultured cells differentiate into the osteoclast lineage. Therefore, these frequencies on the 17th and 22nd day of culture meant that close to all wells contained TRAP$^+$ cells.

The ratio of TRAP$^+$ cells versus total cells in TRAP$^+$-wells was calculated. Mean ratios of TRAP$^+$ cells and TRAP$^+$ MNCs were relatively constant in spite of culture terms (Fig. 3C), but various TRAP$^+$-wells consisted of a well with only one cell, with approximately 1000 cells but 20 TRAP$^+$ cells (Supplementary Fig. S5).

In this section, the culturing term for the final differentiation phase was extended from 6 days. This extension allowed for increased cell numbers in a well and the number of TRAP$^+$-wells. In these cultures, any reagent except for M-CSF and RANKL was not added, suggesting that autonomous regulation may induce an increase in TRAP$^+$-wells.

4. Discussion

In this study, we showed that cloned macrophage-like cell line, C7 derivatives differentiated into TRAP$^+$ osteoclast-like cells, but

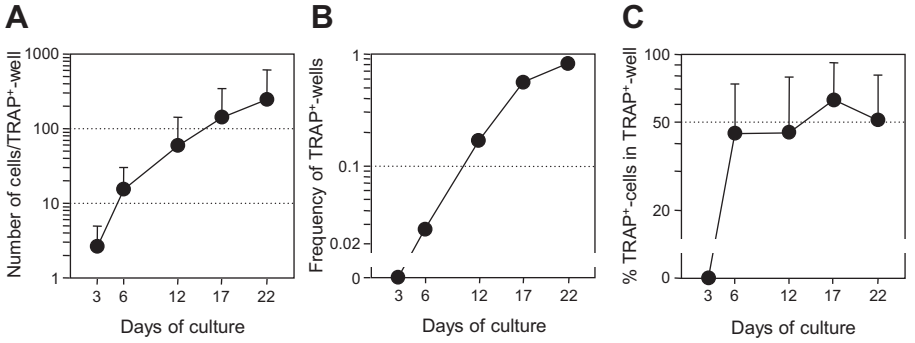

Fig. 3. Cell number in TRAP$^+$-wells and frequency of TRAP$^+$-wells increased with the number of culture days. C7-TY-8 cells pre-cultured with M-CSF for 3 days were harvested and 700 cells were cultured in the presence of M-CSF and RANKL in hybridoma dishes. Every 3 days, half of the medium was changed to fresh medium. On the 3rd, 6th, 12th, 17th, and 22nd day in hybridoma dishes, (A) the total cell number in TRAP$^+$-wells was counted, (B) the number of TRAP$^+$-wells was counted and the frequency of TRAP$^+$-wells was calculated, and (C) the ratio of TRAP$^+$ cells versus total cells in TRAP$^+$-wells was calculated.

only a part of them differentiated and the frequency of differentiation was relatively constant [9]. Although a variety of stimulants were added in 3-day pre-cultures, no reagent could induce significant commitment directed to an osteoclast lineage. Only extending the terms of cultures for osteoclastogenesis increased the ratio of TRAP$^+$-wells.

C7 and its derivatives were repeatedly passed and re-cloned, but their frequencies to differentiate into TRAP$^+$ osteoclast-like cells were less than 0.14 for 6-day cultures. Was the 6-day culture term insufficient for committing C7 cells to differentiate into TRAP$^+$ cells? We previously reported on the kinetics of osteoclastogenesis and showed that C7 and C7-TY cells gave rise to TRAP$^+$-cells including multinucleated cells that resorb dentin slices within 4 days in the presence of M-CSF and RANKL [9,10], and also that even totipotent mouse embryonic stem cells could give rise to TRAP$^+$-cells within 8 days [18,19]. Here, we employed the culture system as follows; 3-day pre-culture for the induction of commitment directed to the osteoclast lineage and a further 6-day culture with M-CSF and RANKL for the final differentiation phase. Under this condition, almost all cells may be able to differentiate into TRAP$^+$-cells within 6 days, if cells have committed to OCPs in the initiation of final differentiation cultures.

Is it because of a specific phenotype of C7 cells that only a small fraction of cells become OCPs? Three cell lines independently established showed a clear consistency of frequencies. BDM-1 cells by Shin et al. [12], and a monocyte line from the SV40 T antigen-transgenic mouse by Chambers et al. gave rise to osteoclast-like cells at a ratio of less than 0.1 [13], which is in the range of that of C7-TY-8 cells, suggesting that these 3 cell lines may be under the same regulation.

Using different kinds of reagents, we stimulated and/or inhibited a variety of signaling pathways of C7-TY-8 cells for 3 days prior to setting the frequency analysis of OCPs. Significant influences in frequency exceeding 0.14 were not reported (Table 1 and Fig. 2). To rule out the possibility that our culture system could not induce a maximal response where all cells give rise to TRAP$^+$ osteoclast-like cells, we used another macrophage-like cell line, RAW264.7, or M-CSF-induced BMMs for OCPs. As shown in Supplementary Table S2, the frequency of BMMs induced by M-CSF and RANKL was 0.452 and that of RAW264.7 cells by RANKL was more than 0.58 (0.588–0.693), indicating that almost all BMMs and RAW264.7 cells gave rise to the osteoclast lineage in 6-day cultures. Therefore, our culture system should function well. A report on human monocytes fractionated from peripheral blood also showed that all cells differentiated into TRAP$^+$ cells [7], suggesting the cell fate of these samples including BMMs and RAW264.7 cells may be decided through a deterministic process by RANKL (and M-CSF).

The proliferation of C7-TY-8 cells slowed down without M-CSF in pre-cultures, but the frequency of TRAP$^+$-wells was not reduced (Table 1), suggesting that times of cell division are not critical for the frequency of precursors for TRAP$^+$ cells. C7-TY-8 cells theoretically started from a single cell in each well of the hybridoma dishes and proliferated there; nevertheless only a part of them differentiated into TRAP$^+$ cells in each well [9,10]. In Supplementary Fig. S5, we showed the number of total cells and TRAP$^+$ cells in TRAP$^+$-wells and that both numbers were extremely scattered.

Results from bulk cultures with 10^4 C7-TY-8 cells or 5×10^3 RAW264.7 cells in a well were impressive. Only some cells were TRAP$^+$ in C7-TY-8 cell cultures, whereas almost all cultured RAW264.7 cells were TRAP$^+$ (Supplementary Fig. S1C and D). Bulk cultures contained enough cell numbers to interact with each other in a well, but results were similar to those from cultures performed in hybridoma dishes initiated from a single cell in a well. The effect of cell concentration in pre-culturing on the frequency of TRAP$^+$ cell generation was not significant (Supplementary Fig. S3). It is likely that the C7-TY-8 cell fate is determined by a cell autonomous mechanism rather than stimulation with cell–cell interaction.

Here, an extension of the culture term for analysis only enabled elevations in the frequency of TRAP$^+$-wells. C7-TY-8 cells proliferated in wells, and on the 22nd day, the average cell number was 245/well (Fig. 3A). At that time, the frequency of TRAP$^+$-wells was 0.831, indicating that nearly all wells with C7-TY-8 cells presented contained TRAP$^+$ osteoclast-like cells. The same event should be repeated in the individual wells of hybridoma dishes. The event where C7-TY-8 cells differentiated into TRAP$^+$ cells occurred at a constant frequency, less than 0.1. However, the reason why nearly 50% of cultured cells stained for TRAP activity is not clear (Fig. 3C). In this culture condition, TRAP$^+$ cells may grow faster or survive more efficiently than TRAP-negative cells. In wells containing hundreds of cells, TRAP activity seemed to be delivered to neighboring cells, resulting in a higher ratio of cells estimated as TRAP$^+$ cells. We cannot currently explain this mean ratio of TRAP$^+$ cells in the well, but it does not conflict with our recent conclusion that only extended cultures accelerate the emergence of TRAP$^+$-wells.

Recently, Jaenisch and his colleagues claimed that reprogramming of somatic cells into induced pluripotent stem (iPS) cells contains a continuous stochastic process, and that distinct cell-division-rate-dependent and -independent modes for accelerating the stochastic course of reprogramming are present [17,20]. They demonstrated the stochastic/probabilistic phase and deterministic/hierarchical phase as models for the progression to a pluripotent state during direct reprogramming. Although the direction of differentiation is opposite, our observation for the temporal frequency of TRAP$^+$ cells from C7-TY-8 with cell growth seems to coincide with their stochastic phase (Fig. 3). The graph in Fig. 3B shows that increased frequency with culture days drew an upward slant line chart as a stochastic process, but not a deterministic stepped line chart.

The regulation of this "stochastic process" is currently unknown, but we believe our model provides a novel mechanism for the decision of cell fate including stem cell biology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.052.

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