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Osteoclast fusion is initiated by a small subset of RANKL-stimulated monocyte progenitors, which can fuse to RANKL-unstimulated progenitors

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ABSTRACT

Osteoclasts are multinucleated, bone-resorbing cells formed via fusion of monocyte progenitors, a process triggered by prolonged stimulation with RANKL, the osteoclast master regulator cytokine. Monocyte fusion into osteoclasts has been shown to play a key role in bone remodeling and homeostasis; therefore, aberrant fusion may be involved in a variety of bone diseases. Indeed, research in the last decade has led to the discovery of genes regulating osteoclast fusion; yet the basic cellular regulatory mechanism underlying the fusion process is poorly understood. Here, we applied a novel approach for tracking the fusion processes, using live-cell imaging of RANKL-stimulated and non-stimulated progenitor monocytes differentially expressing dsRED or GFP, respectively. We show that osteoclast fusion is initiated by a small (~2.4%) subset of precursors, termed "fusion founders", capable of fusing either with other founders or with non-stimulated progenitors (fusion followers), which alone, are unable to initiate fusion. Careful examination indicates that the fusion between a founder and a follower cell consists of two distinct phases: an initial pairing of the two cells, typically lasting 5–35 min, during which the cells nevertheless maintain their initial morphology; and the fusion event itself. Interestingly, during the initial pre-fusion phase, a transfer of the fluorescent reporter proteins from nucleus to nucleus was noticed, suggesting crosstalk between the founder and follower progenitors via the cytoplasm that might directly affect the fusion process, as well as overall transcriptional regulation in the developing heterokaryon.

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Introduction

Physiological skeletal homeostasis is a well-coordinated process, regulated by the reciprocal actions of bone-forming osteoblasts and bone-resorbing osteoclasts [1,2]. The fine balance between these two activities is of critical physiological importance, since decreased bone resorption by osteoclasts leads to the formation of sclerotic bone, as seen in osteopetrosis, whereas excessive resorption drives the pathogenesis of osteoporosis, osteoarthritis, periodontal diseases, tumor metastasis into bone, and multiple congenital syndromes [3]. Thus, an understanding of the mechanisms that control the activity of osteoclasts is crucial to the diagnosis and treatment of many clinical conditions.

Osteoclasts are large, multinucleated cells derived from the monocyte/macrophage lineage, and their differentiation requires factors produced by marrow stromal cells, osteoblasts, osteocytes, or lymphocytes [4–7]. Two pivotal factors, necessary and sufficient to promote osteoclastogenesis, are the macrophage colony-stimulating factor (CSF-1), and the receptor activator of nuclear factor kappa B ligand (RANKL) [8,9]. CSF-1 supports osteoclast proliferation and survival, whereas RANKL induces activation of the osteoclast differentiation program, including activation of genes necessary for bone resorption, and genes required for fusion of monocyte progenitor cells. Cell fusion is an essential step in osteoclast development, shared only by a few other cell types such as skeletal myoblasts, trophoblasts in the placenta, and the egg and sperm [10–12]. Given its central physiological role, osteoclast fusion may serve as an attractive target for specific therapeutic intervention in bone diseases where the action of these cells is either excessive or deficient.

In recent years, several studies utilized mouse models and cell-culture approaches to address the molecular mechanisms underlying osteoclast fusion, identifying proteins such as DC-STAMP [13,14], CD47 [15,16], dynamin [17], OC-STAMP [18] and ATP6v0d2 [19] (see also review [20]), that are essential to this process. At the cellular level, however, the exact role of each of these molecules in the fusion process remains elusive.

In this study, we addressed the properties and heterogeneity of osteoclast monocyte progenitors participating in the generation of the polykaryon. For this purpose, we examined the kinetics and fusion

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patterns of osteoclast progenitor cells, and developed a novel approach for tracking individual cells involved in the initiation of the fusion process, and its progression. Using RAW 264.7 cells tagged with different fluorescent markers, we demonstrated that only a small subset of "founder cells" within the population of RANKL-stimulated cells was capable of initiating the fusion process, following 48–72 h of stimulation. When RANKL-primed (i.e., cells incubated for 48 h with RANKL) RAW264.7 cells were mixed with unprimed RAW264.7 cells, we found that the unprimed RAW cells could fuse with a primed founder cell. Moreover, live-cell experiments indicated that as soon as a founder progenitor cell and a follower progenitor cell adhere to each other, they develop cytoplasmic communication, well before the fusion event becomes apparent. The significance of the heterogeneity of the progenitor cell population and the mode of cell–cell interaction involved in the fusion process are discussed.

Materials and methods

Generation of RAW264.7 clones expressing nuclear fluorescence proteins

To generate RAW264.7 clones, cassettes expressing nuclear fluorescence proteins DsRed and AceGFP containing nuclear localization signals from pDsRed2-NUC and pAcGFP-NUC, respectively [Clontech], were amplified by PCR, introducing NotI and ClaI restriction sites. The PCR product was cloned into the lentiviral vector pHAGE2-Full EF1a-DsRedExpress-IRES-ZsGreen-W (courtesy of Dr. Roi Gazit) by replacing the DsRED-IRES-Zs Green cassette, using the corresponding restriction enzymes. For the productions of virions, lentiviral vectors expressing the different nuclear fluorescent proteins were packaged in HEK293T cells, using the packaging plasmids pMD2.G and psPAX2 (Addgene). The virions were collected 48 h after transfection. RAW264.7 cells were infected for 48 h in the presence of 8 μg/ml polybrene (Sigma). Clones expressing different fluorescent nuclear proteins were selected in puromycin (3 μg/ml) for 3 days.

Isolation of bone marrow monocytes

Cells were harvested from the bone marrow of C57BL6 mice, as previously described [21]. Bone marrow cells were treated with ACK red cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM Na_2EDTA in distilled H_2O). Cells were plated on bacterial culture dishes in α-MEM and 10% FBS for three days in the presence of recombinant CSF-1 (20 ng/ml) (R & D Systems), and then stained and used in co-culture experiments.

Osteoclast fusion distribution analysis

To calculate the distribution of fusion events in which either mononucleated or multinucleated cells were involved, RAW264.7 cells expressing actin-GFP or BMMs were plated in wells of μclear 384-well plates at a density of 1000 cells per well, and cultured in α-MEM containing 10% FBS, 20 ng/ml murine recombinant CSF-1, and 20 ng/ml or indicated concentrations of RANKL. After 48 h in culture, medium was replaced, and cells were cultured for an additional 72 h, during which they were monitored by time-lapse microscopy. To compare fusion of mononucleated and multinucleated cells, fusion events were counted in a total of 60 movies from three independent experiments. A total of 1098 fusion events were counted in these experiments, and plotted against time. To compare mononucleated cell fusion in the presence of different concentrations of RANKL, fusion events were counted in ten movies for each RANKL concentration. The numbers of fusion events/field/cell were also quantified. To monitor founder cell distribution, RAW264.7 clones expressing nuclear AceGFP were plated in 250 μm-wide wells of CytoCapture dishes (Mobitec), by plating a total of 100,000 cells per dish. Cell density in each well ranged between 12 and 120 cells per well. After 72 h of culture in the presence of CSF-1

and RANKL, cells were imaged, and the total number of cells and multi-nucleated cells with three or more nuclei within each well was counted.

Founder-follower co-cultures

For generation of "founder"-enriched cultures, RAW264.7 clones expressing dsRED were plated in wells of μclear 384-well plates, at a density of 1000 cells per well. The cells were cultured in α-MEM containing 10% FBS, and 20 ng/ml murine recombinant CSF-1 with 20 ng/ml RANKL (Founders), or without RANKL (Control). RAW264.7 clones expressing nuclear AceGFP were cultured in α-MEM containing 10% FBS and 20 ng/ml murine recombinant CSF-1 without RANKL; these served as followers. After 50 h of incubation, follower cells were lifted off the plates, and 1000 cells were transferred to wells containing either DsRED founder or control cells. The co-cultured cells were cultured in the presence of CSF-1 and RANKL for a duration of 12 h, during which they were monitored by time-lapse microscopy.

For BMM co-culture experiments, BMMs were separated into two groups. One group was stained with the cytoplasmic dye PKH67, and the second group with Cell Vue Claret dye, according to manufacturer's protocols (Sigma). Cell Vue Claret-stained BMMs were plated in wells of μclear 384 well plates at a density of 1000 cells per well, and cultured in the presence of 20 ng/ml CSF-1, with 20 ng/ml RANKL (Founders) or without it (Control). BMMs stained with PKH67 dye were cultured only in the presence of CSF-1, and served as the follower group. Founder and Control BMMs were cultured for 72 h, after which 1000 follower cells were added to each well. The co-cultured cells were cultured in the presence of CSF-1 and RANKL for 12 h, during which they were monitored by time-lapse microscopy.

Microscopy

Movies and fixed cell data were acquired with a live imaging system, consisting of an inverted IX81 microscope equipped with 20×/0.75 NA objectives (Olympus) and with a temperature-controlled box using CellSens software (Olympus). Nuclear fluorescence transfer was calculated in the following way: nuclei segmentation and the measurement of total nuclei intensity were performed by means of ImageJ software. The initial green fluorescence in the red nuclei was set as "background," and subtracted from the green fluorescence readings at each time point. To normalize the results to the initial fluorescence level of the green nuclei, the green fluorescence readings in the red nuclei at each time point were divided by the initial green fluorescence of the green nuclei, minus the background green fluorescence.

Immunofluorescence

Three clones of RAW264.7 cells were seeded on coverslips and induced to differentiate using the conditions outlined above. Cells were fixed at 48 h of differentiation in 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton X-100 and blocked with PBS containing 2% FBS for 1 h. NFATc1 expression was detected using overnight incubation in NFATc1 mAb (1:100, Santa Cruz Biotechnology) and four hours' incubation with Cy2-conjugated antimouse secondary antibody (AffiniPure, Jackson). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Nuclei and cytoplasmic fluorescence intensities were quantified using ImageJ, and normalized to background levels.

Statistical analysis

The probability picking founders in a cell population is governed by Poisson statistics, but can be well approximated by normal (Gaussian) statistics for large numbers of cells per sample (50 ± 20 for our experiment). We counted the initial number of cells and listed the number of fused cells in 142 samples. We plotted the distribution of x, the

number of founders per seeded cell for these samples, and fitted this curve to the analytical expression for Gaussian statistics of the probability distribution to find more than x founders namely, $A[1 - \text{erf}(x / B)]$, where B is the fraction of founder cells, yielding $B = 0.024$. Thus, the percentage of founder cells in a population of differentiating RAW264.7 cells is ~2.4%. This result assumes that each fused osteoclast originates from one founder cell. The small fraction of founders justifies neglecting the rare events of two or more founders in a single fused osteoclast.

Results

Osteoclast fusion is initiated by a limited and rare subset of osteoclast progenitors

In order to track the cellular characteristics of osteoclast fusion, we used live-cell fluorescence microscopy to monitor the fusion process of RANKL/CSF-1-stimulated RAW264.7 cells expressing GFP–actin. Upon stimulation, this monocyte/macrophage cell line forms giant osteoclast-like polykaryons [22]. The expression of GFP-tagged actin in these cells enabled us to easily monitor actin ring formation, which assembles following the initiation of cell fusion, and is believed to correspond to the "sealing zone" formed by osteoclasts adhering to the physiological bone surface.

Initial fusion events of cultured RAW264.7 cells are observed after 48 h of incubation with RANKL; within 72 h, the culture is dominated by giant osteoclast-like cells. Live-cell monitoring of RANKL-treated cultures indicated that even though all cells were exposed to the same culture conditions, the first fusion events were initiated by only a small subset of the cells. The new binucleated cells thus formed continued to fuse to other cells, increasing the size of the polykaryon, and the diameter of its expanding sealing zone (Fig. 1A, Movie 1). These observations indicated that the rate at which fusion of multinucleated cells progresses, is faster than the rate of initial fusion events between two mononucleated RAW264.7 cells. Quantification of the relative prominence of fusions between two polykaryons, between a polykaryon and a mononucleated cell, or between two mononucleated cells, suggests that the initial fusion event depends on a rare population of fusion-founder precursors (Fig. 1B). This rare subset of founder precursors did not change significantly, even when the osteoclast progenitor cultures were exposed to elevated concentrations of RANKL (Fig. 1C).

What is the mechanism whereby the small population of founder cells is generated? One possibility is that the RANKL-induced transformation of osteoclast precursors into committed founder cells is a poorly synchronized process, and only some of the stimulated RAW264.7 population gains fusion competence within 48–72 h of stimulation. Alternatively, osteoclast precursors may be intrinsically heterogeneous, comprising founder and follower subpopulations in which only the small subset of founder cells displays fusion-initiation competence. In the absence of specific molecular markers for the "founder state", the cell culture conditions used in these experiments cannot unequivocally distinguish between the two possibilities described above.

To address this issue, we cultured RAW264.7 cells in microwells in which the number of cells per well was systematically controlled, and cell fate, continuously monitored. This culture system enabled us to estimate the frequency of founder cells when cells are exposed to the same culture conditions, but separated into small groups ranging from 10 to 120 cells, and placed in 160 μm, hexagonal microwells (Fig. 1C). To maximize the probability that the cells would express their fusogenic capacity, they were incubated for 96 h in the presence of RANKL and CSF-1, and both the total numbers of cells, and the numbers of multinucleated, osteoclast-like cells, were counted in each well. Multinucleated osteoclasts representing founder cells ranged from 0 to 3 per well, indicating that only approximately 2.4% of the cells are potent fusion founders (see detailed explanation of the statistical analysis in the Materials and methods section). These results suggest that osteoclast

precursors are intrinsically heterogeneous, comprising a small subset of founder cells and a majority of "followers". To test whether this heterogeneity is a result of different osteoclast differentiation states, we monitored the expression and localization of NFATc1, a master transcription regulator of osteoclast differentiation that is essential for fusion of osteoclast precursors [23]. In its inactive state, NFATc1 is located in the cytoplasm and upon activation, translocates to the nucleus to initiate transcription of target genes. In osteoclasts, NFATc1 translocation to the nucleus is usually observed 48 h after the cells are induced to differentiate by the addition of RANKL to the culture. This translocation event is followed by an increase in NFATc1 expression as a result of transcription auto-regulation [24]. To determine if any given population of RAW264.7 cells is heterogeneous for the cell differentiation states, we induced differentiation of RAW264.7 clones derived from single cells. Immunofluorescence staining in cells induced to differentiate for 48 h showed heterogenic expression and localization of NFATc1 in the cell population (Fig. 1E). Quantification of NFATc1 expression and localization in differentiation cultures of three different RAW264.7 clones showed scattered expression, with low expression in the majority of cells. Taken together, our results show that RAW264.7 progenitor cells are heterogeneous for the expression of differentiation, and only a rare, limited subset of differentiating progenitor cells acquires the capacity to initiate osteoclast fusion.

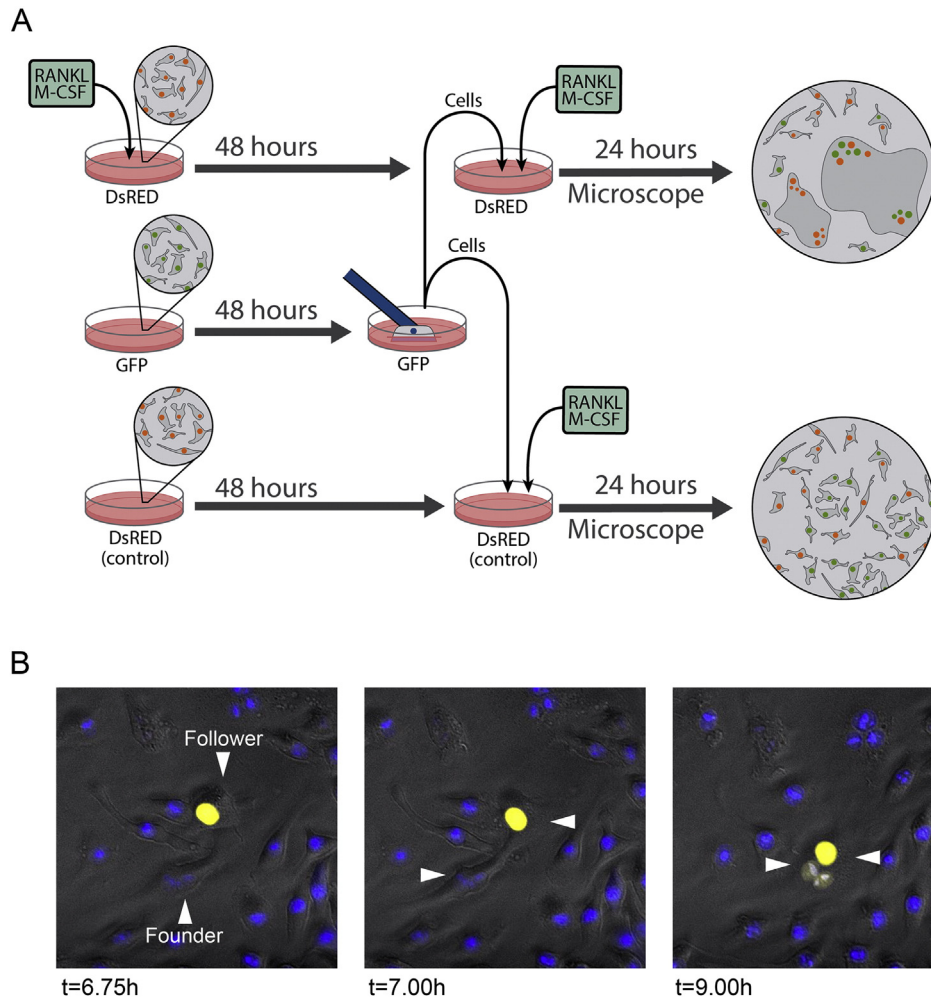### Fusion founder cells can fuse with naïve osteoclast precursors devoid of fusion-initiation capability

Given that only a small subpopulation of RAW264.7 cells actually serves as fusion founders, while the overall population of cells that participate (as "followers") in the fusion process is considerably larger, we directly examined whether fusion follower cells require extended exposure to RANKL, in order to fuse to founder cells. To explore this possibility, RAW264.7 cells expressing nuclear dsRED were cultured in the presence of CSF-1, with or without RANKL. After 50 h of incubation, RAW264.7 cells expressing AceGFP that were cultured in the presence of CSF-1 without RANKL were mixed with the dsRED-expressing cells, and fusion was monitored by time-lapse microscopy (Fig. 2A). In mixed cultures, in which dsRED cells were not pre-exposed to RANKL, no fusion was detected in 20 frames containing 2000 cells, for the duration of 12 h, even when RANKL was added to the co-culture. These observations indicated that extended exposure to RANKL is required for fusion to take place, consistent with previous reports showing that fusion of RAW264.7 cells cultured in CSF-1 and RANKL is initiated only after two days or more in culture. On the other hand, fusion of dsRED-expressing cells to AceGFP-expressing cells was readily detected in the mixed cultures, in which only the dsRED-expressing cells were previously exposed to RANKL. This finding indicates that participation in fusion events as followers does not require long exposure to RANKL. In fact, fusion events in these co-cultures were already detected within 3 h after the cells were mixed (Fig. 2B and Movie 2). It was further noticed that fusion of AceGFP-expressing cells among themselves was not observed in these mixed cultures, indicating that cells not pre-exposed to RANKL cannot serve as fusion founders. In conclusion, these results confirm the hypothesis that prolonged exposure to RANKL is essential for acquisition of an osteoclast-fusion founder cell state, but is dispensable for the acquisition of fusion follower state in cultured RAW264.7 cells.

Primary bone marrow-derived pre-osteoclasts adopt a founder–follower mode of fusion

In order to test whether the osteoclast founder–follower mode of fusion exists in a more physiologically relevant system, we monitored the fusion distribution of osteoclast precursors in cultures of isolated murine bone marrow monocytes (BMMs). As in the RAW264.7 cell cultures, analysis of fusion distribution using time-lapse microscopy showed that the rate of fusion into multinucleated cells in BMM cultures
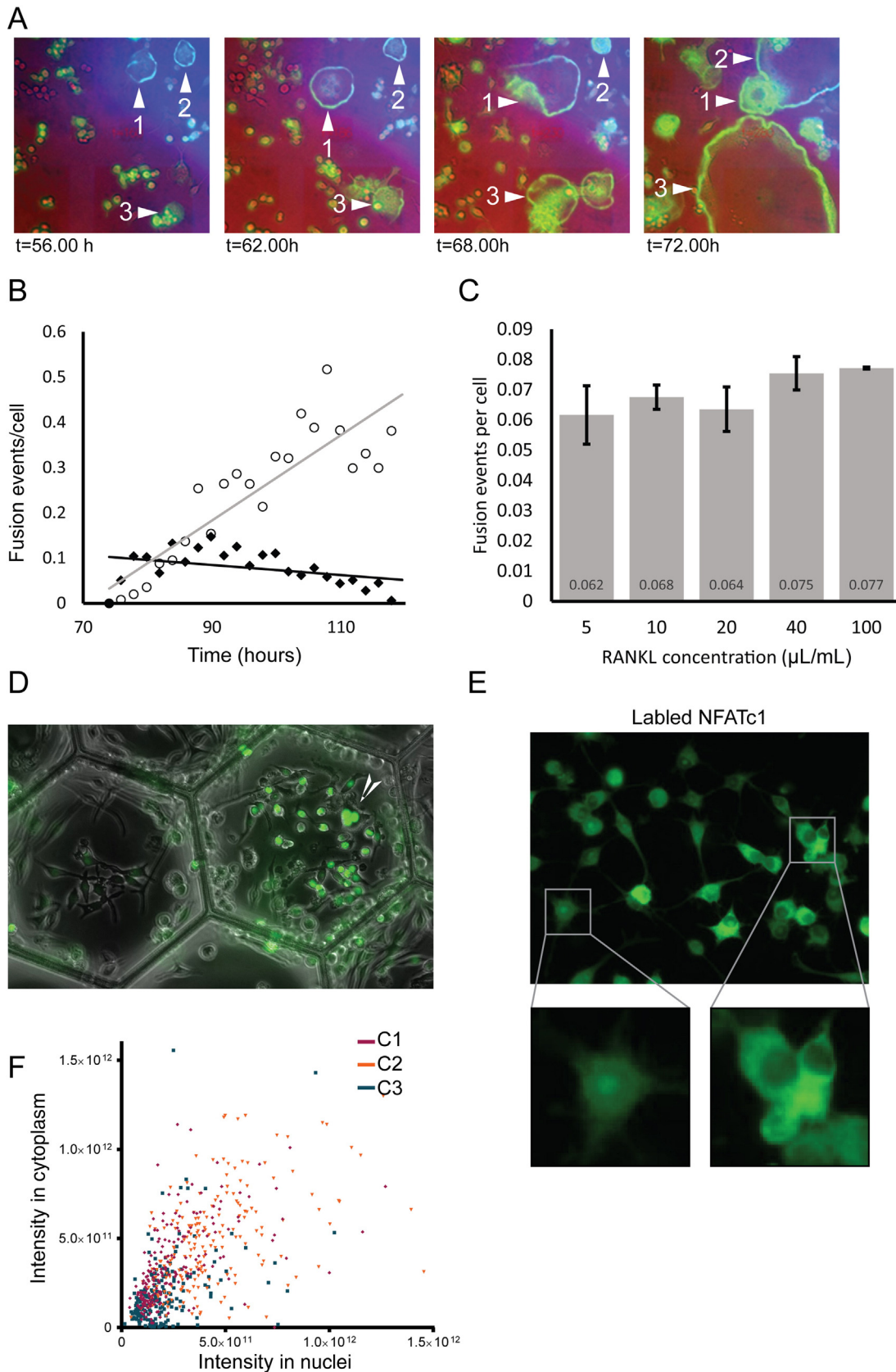
Fig. 1. Osteoclast fusion is initiated by a rare, limited subset of fusion founders. (A) Images of RAW264.7 clones expressing actin-GFP cultured in the presence of RANKL and CSF-1. The images represent a sequence of fusion initiation and progression of three different cells (marked by white arrows) over 16 h, until giant, multinucleated cells are observed. (B) The distribution of fusion events in which both fusion partners are mononucleated cells (empty circle) or at least one of the cells is a multinucleated cell (black diamond) was quantified, and plotted against time. (C) Mononucleated cell fusion events of RAW264.7 cells cultured in the presence of the indicated RANKL concentrations were quantified and normalized to total cell numbers. (D) An image showing RAW264.7 clones expressing nuclear AceGFP, and cultured on micro-wells in the presence of RANKL and CSF-1. A multinucleated cell is denoted by a white arrowhead. (E) Immunofluorescence staining of NFATc1 in cultures of differentiating RAW264.7 cells. Image enlargement demonstrates different NFATc1 intensities and localizations in different cells. (F) NFATc1 cytoplasmic fluorescence intensity was plotted against its nuclear fluorescence intensity in RAW264.7 cultures derived from three different single-cell clones.

Fig. 2. RANKL-primed osteoclast precursors can fuse with unprimed osteoclast precursors devoid of fusion initiation capabilities. (A) Schematic presentation of the co-culture method used to test whether RANKL-pretreated founder cells can fuse with untreated cells. (B) Images showing a RANKL primed RAW264.7 cell expressing nuclear DsRed (pseudo-colored blue), fusing with an unprimed RAW264.7 cell expressing AceGFP (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was significantly higher than mononucleated cell fusion, suggesting a "founder-follower" mode of fusion (Fig. 3A).

We further examined, in primary osteoclast precursors, whether heterotypic osteoclast cell fusion could be regulated by RANKL exposure. For this purpose, isolated BMMs were divided into two groups. One group was stained with the cytoplasmic dye PKH67, and the other group, with Cell Vue Claret. Similar to the RAW264.7 cells, Cell Vue Claret-stained BMMs were cultured in the presence of CSF-1 with RANKL (Founders), or without it (Control). BMMs stained with PKH67 dye were cultured in the presence of CSF-1 only, and served as the follower group. Since fusion initiation in cultures of BMMs requires longer incubation than in RAW264.7 cells, putative founder BMMs were cultured for 72 h in the presence of RANKL, before being mixed with the follower BMMs. No spontaneous fusion of cells was observed in cultures of followers that had not been pretreated with RANKL. In contrast, instances of founder-follower cell fusion were readily detected in mixed cultures in which the founders were pretreated with RANKL (Fig. 3B, Movie 3). Taken together, these results show that distribution of fusion events in primary osteoclast precursors adopts a founder-follower mode, and that RANKL-primed primary osteoclast precursors can fuse with BMMs lacking independent fusion-initiating capabilities.

Founder and follower cells establish cytoplasmic communication before membrane fusion is apparent

Our results thus far suggest that osteoclast fusion is mediated by a rare subset of cells, which both initiate and lead the fusion process. Our approach of labeling the nuclei of fusion partners with different fluorescent proteins enabled the tracking of fusion partners before, during and after fusion took place. Beyond the use of this two-color system for distinguishing between the founder and follower cell populations, it enabled us to determine the exact time point during the interaction of the initial pair of founder and follower cells, at which cytoplasmic communication was established between the two cells. The rationale underlying this experiment was that fluorescent proteins are thought to shuttle between the cytoplasm and the nucleus. When cytoplasmic communication occurs, the originally green or red nuclei are expected to begin importing the fluorescent reporter produced by the partnering cell.

In a series of preliminary experiments, we clearly observed entry of AceGFP into the red nucleus of the associated founder cell, though the entry of the DsRed protein into the partnering cell was considerably slower (Fig. 4A and Movie 4). Tracking the fusion of osteoclast precursors by time-lapse microscopy revealed several steps in the first fusion event. In the initial step, fusion partners migrated toward each other,
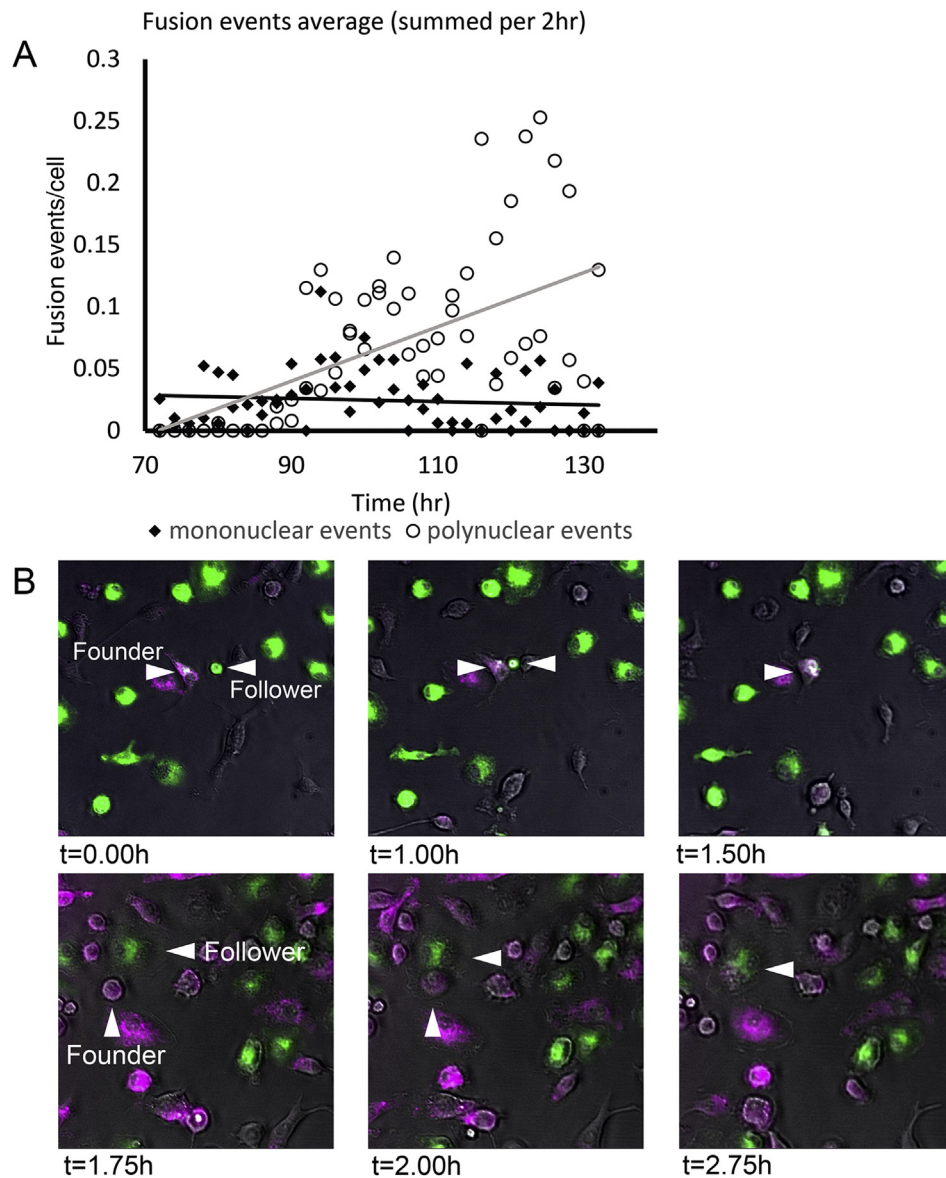
Fig. 3. Primary bone marrow pre-osteoclasts adopt a founder-follower mode of fusion. (A) BMMs were cultured in the presence of RANKL and CSF-1, and monitored by time-lapse micros-copy. The distribution of fusion events of differentiating BMMs in which both of the fusion partners were mononucleated cells (diamond), or at least one of the cells was a multinucleated cell (empty circle), was quantified and plotted against time. (B) Images showing RANKL-primed BMM stained by Cell Vue Claret (purple) fusing with unprimed BMM stained by PKH67 (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and appeared to adhere to each other without changing their overall rounded morphology. Typically, the "adhesion step" lasted between 5 and 35 min; a scant few minutes later, membrane fusion was visible, followed shortly thereafter by the migration of nuclei of the fused cells toward the multinucleated cell center (a process that usually lasted 10 min). Interestingly, analysis of AceGFP import into the red founder cell nucleus showed that the color was transferred between the cells, long before membrane fusion was visible by phase contrast microscopy (Fig. 4B). These observations suggest that when founder and follower cells interact with each other, they establish communication channels (see Discussion) through which proteins can be exchanged between the cytoplasms of the founder and follower cells. The increase in the level of AceGFP in the founder cell nucleus over time is linear, and appears to begin as soon as the two cells adhere to one another.

Discussion

In this study, we focused on the cellular mechanisms underlying the fusion of monocyte progenitors into osteoclasts, multinucleated, bone-resorbing cells that play a critical role in bone remodeling and homeo-stasis. Utilizing novel approaches to study osteoclast differentiation, we demonstrated that osteoclast fusion is initiated by a small subset of osteoclast precursors. Our data indicate that only about 2.4% of a population of RAW264.7 cells stimulated by RANKL and CSF-1 can ac-quire fusion founder competence; moreover, the percentage of these cells remains below 10%, even when the cells are exposed to increased concentrations of RANKL, or longer incubation times. In addition, we showed that while prolonged exposure to CSF-1 and RANKL is essential to induce the fusion of founder cells, it is not required for the subsequent fusion of follower cells to them. Furthermore, we found that during the initial stages of fusion, proteins are transferred between founder and follower cells. Hence, it is possible that in early stages of fusion, diffus-ible cytoplasmic molecules are exchanged between founder and follow-er cells. This notion is in line with earlier studies showing that in early stages of osteoclastogenesis, fusion-competent protrusions are formed, interconnecting the fusing cells [25]. Whether such a cytoplasmic exchange process plays a critical role in the fusion event itself remains to be determined.
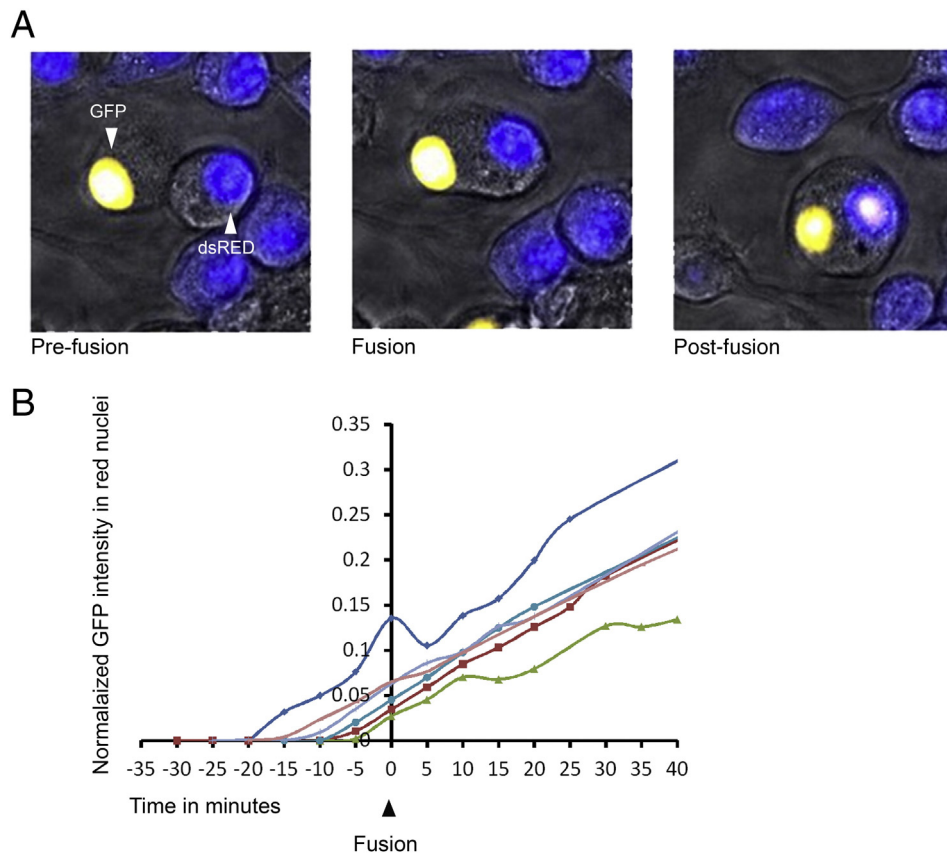
Fig. 4. Proteins are transferred between founder and follower cells at initial stages of the fusion process. (A) Images showing transfer of AceGFP (yellow) from a nucleus of an osteoclast precursor expressing nuclear AceGFP, to the nucleus of another precursor expressing DsRED during the fusion process. (B) A graph showing the gradual increase of AceGFP intensity in DsRED-expressing nuclei measured before membrane fusion was visible (negative time points), during membrane fusion (time point zero), and after the generation of multinucleated cells (positive values). Six representative fusion events are plotted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

It is worth noting that the terms fusion-founder and fusion-competent cells were used in studies of myogenesis in *Drosophila*, where myoblast fusion "founder cells" are fusing with "fusion-competent cells" [26]. Several observations reported in the literature support the existence of a cellular heterotypic osteoclast fusion model in vitro. Osteoclasts and the giant macrophage cells share a common progenitor. Although fusion of osteoclasts is induced by RANKL and macrophage formation is induced by IL4 [27–29], both osteoclasts and macrophages share the expression of multiple fusion-regulating genes, suggesting a common fusion-regulating mechanism [14,15,19,30–33]. Helming and Gordon showed that fusogenic macrophage populations can cross-fuse with non-fusogenic macrophage populations, suggesting that macrophage fusion and hence osteoclast fusion may be cellular heterotypic [34]. Additional evidence supporting heterotypic osteoclast fusion comes from findings that mice BMMs lacking expression of the fusion-essential genes DC-STAMP and OC-STAMP, as well as ATP6v0D2, can fuse to wild-type osteoclasts [14,18,19].

Our study is the first to demonstrate that the abundance of cells acquiring fusion-founding capabilities among a monocyte population is rare, and limited. Notably, this limitation on the number of cells that can acquire founding competency was maintained, despite the fact that homogenous populations of RAW264.7 cells were used. Even when RAW264.7 cell cultures were generated from single clones, the percentage of fusion founder cells remained below 10%. A possible explanation for the heterogeneity of these cultures with regard to fusion founder capabilities could be derived from the fact that these cells are monocyte precursors with the ability to differentiate into a number of cell types, such as macrophages and dendritic cells. Thus, at any given time, a population of RAW264.7 cells could be heterogeneous, consisting of cells at a number of stages of differentiation toward various cell fates. This assumption is supported by our observation that after 48 h of differentiation, RAW264.7 cell populations derived from single clones show diverse expression and localization of the osteoclast transcription "master regulator", NFATc1. It was shown that as osteoclasts differentiate, NFATc1 translocates to the nucleus to activate osteoclast regulatory genes, as well as its own transcription. Therefore, advanced differentiation of osteoclast precursors is marked by the increased expression and nuclear localization of NFATc1. Our observation showing non-uniform expression and localization of NFATc1 suggests that at least in this respect, osteoclast cultures derived from single cell clones consist of monocyte progenitors at different stages of differentiation. Further support for heterogeneity in cultures of osteoclast progenitors comes from recent findings that human blood monocyte cells express different levels and subcellular distributions of fusion-regulating genes such as CD47, DC-STAMP, and Syncytin-1 [35]. Moreover, it was shown that the commitment of RAW264.7 monocyte progenitors to active macrophage differentiation involves suppression of their differentiation toward an osteoclast fate [36]. Thus, the restriction on acquiring fusion-founding competence could be a result of commitment to another cell fate. Nevertheless, the fact that fusion founders can fuse with followers devoid of fusion initiation competence suggests that once a fusion founder is formed, it can fuse with monocytes directed to other cell fates.

This observation, however, begs the question: what restricts the differentiation of follower into founder cells? To address this issue, we incubated RAW264.7 cells with the NIH3T3 fibroblast cell line and observed no fusion between the two cell types, indicating that there is a restriction on the identity of the follower cell. Moreover, in preliminary

studies, we generated RAW264.7 clones in which the RANK receptor was knocked out using the CRISPR/CAS9 system. These clones could not fuse to RAW264.7 cells expressing RANK. Thus, stimulation by RANKL is essential for both fusion founder and fusion follower cells, whereas prolonged exposure to RANKL is essential only for the establishment of founder capabilities.

Our data further reveal a novel cellular mode of regulation for osteoclast differentiation, in which a small subset of mature progenitors with fusion-founding capabilities can fuse to less mature osteoclast progenitors for the purpose of generating multinucleated osteoclasts. This mode of fusion may have many important consequences for the physiological role of this process in health and disease, but raises many fundamental questions, such as: (i) what are the molecular and cellular characteristics of fusion founder and fusion follower cells? (ii) does a unique, predetermined subset of osteoclast fusion founder precursors exist? (iii) does fusion rapidly "turn on" the osteoclast transcription program in undifferentiated follower cells? and (iv) is an abnormal distribution of founder and follower cells seen in the various bone pathologies?

Future studies aimed at answering these questions will increase our understanding of osteoclast regulation, and may reveal new molecular targets, with therapeutic implications.

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Acknowledgments

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