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Foreign Body Giant Cells and Osteoclasts Are TRAP Positive, Have Podosome-Belts and Both Require OC-STAMP for Cell Fusion

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ABSTRACT

Macrophages have the ability to fuse and form multinucleated giant cells such as Osteoclast (OCs) and FBGCs. Osteoclast stimulatory transmembrane protein (OC-STAMP) is an important cell surface protein involved in the formation of OCs. This study sought to determine if OC-STAMP also regulates formation of FBGCs using expression analysis and subsequent inhibition studies. qPCR and Western blot analysis showed that OC-STAMP expression is significantly higher in FBGCs compared to control monocytes ($P < 0.05$). Four days following cell culture, OCs were positive for TRAP and F-actin ring formation, but FBGCs were not. In contrast, FBGCs were positive for TRAP and showed podosome belts comprised of F-actin on Day 8. FBGCs were subsequently plated onto dentine, but despite presenting some morphologic features of OCs (OC-STAMP expression, TRAP reactivity, and podosome belts) they failed to resorb bone. To evaluate a role for OC-STAMP in FBGCs, we inhibited this cell surface protein with anti-OC-STAMP antibody and observed that cell fusion and podosome belt formation was inhibited in both OCs and FBGCs. Our data support the hypothesis that OC-STAMP is a regulatory molecule for FBGCs; and that they are functionally distinct from OCs, despite similarities in gene expression profile, podosome belt formation, and TRAP expression. J. Cell. Biochem. 114: 1772–1778, 2013.

KEY WORDS: FBGC; OC-STAMP; DC-STAMP; OSTEOCLAST; MULTINUCLEAR GIANT CELLS

Osteoclasts (OCs) are multinucleated, bone resorbing cells, that are essential for bone remodeling. Osteoclast precursors are mononuclear cells derived from bone marrow. They circulate in blood and are induced in the presence of two cytokines, macrophage colony stimulating factor (MCSF), and receptor activator of nuclear factor kappa-B ligand (RANKL), to form multinucleated cells with a capacity to resorb bone [Boyle et al., 2003]. Activation of the MCSF receptor is necessary for proliferation and survival of osteoclast progenitor cells [Dai et al., 2002]. RANKL is a member of the tumor necrosis factor family (TNF) that leads to activation of the receptor, RANK, present on osteoclast precursors [Dougall et al., 1999]. Mutations of RANK or RANKL block differentiation of osteoclast precursors into mature OCs, resulting in osteopetrosis [Sobacchi et al., 2007]. Activation of OCs by RANKL initiates cytoskeletal changes in OCs resulting in reorganization of filamentous actin (F-actin) around the periphery in the form of a belt or ring, the podosome belt facilitating adhesion [Destaing et al., 2011] and movement of OCs [Hu et al., 2011]. Furthermore, OCs show intense staining for tartrate resistant acid phosphatase (TRAP), which is considered adequate to differentiate OCs from other monocyte derived cells [Burstone, 1959]. Fusion of monocyte/macrophage lineage cells is a complicated process involving different cell surface receptors such as dendritic cell-specific transmembrane protein (DC-STAMP) [Yagi et al., 2005] and OC-STAMP [Yang et al., 2008]. OC-

Abbreviations: OCs, osteoclasts; FBGCs, foreign body giant cells; MCSF, macrophage colony stimulating factor; GMCSF, granulocyte macrophage colony stimulating factor; IL4, interleukin 4; TRAP, tartrate resistant acid phosphatases; RANKL, receptor activator of nuclear factor kappa B ligand; OC-STAMP, osteoclast stimulatory transmembrane protein; DC-STAMP, dendritic cell-specific transmembrane protein; MNGCs, multinucleated giant cells.

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STAMP is a transmembrane protein that is highly upregulated during osteoclast formation. Expression of OC-STAMP increases osteoclast formation, and its inhibition abrogates osteoclast formation and function [Yang et al., 2008].

Bone marrow derived monocyte/macrophage cells also have the capacity to fuse under the influence of IL-4 to form foreign body giant cells (FBGCs) [McNally and Anderson, 1995]. IL-4 also inhibits osteoclast formation from monocyte/macrophage cells, promoting differentiation into cell types such as FBGCs [Bendixen et al., 2001]. Furthermore, granulocyte macrophage colony stimulating factor (GMCSF), a growth factor important for macrophage survival [Ujihara et al., 2001; Kim et al., 2005], has interesting effects on OCs. Short-term exposure of osteoclast progenitors to GMCSF leads to enhanced osteoclast formation, while long-term exposure leads to attenuation of osteoclast formation [Hodge et al., 2004]. In contrast, formation of FBGCs is dependent on the presence of both IL-4 and GMCSF. Neutralizing antibodies against IL-4 and GMCSF significantly reduce macrophage fusion during the formation of FBGCs [McNally and Anderson, 1995]. FBGCs are usually found at the interface of tissue and materials such as prostheses, implants, and medical devices where they play an active role in the foreign body reaction and hinder the osteointegration of various implants [Wu et al., 2011], but also mediate total rejection of the medical devices [Jones, 2008]. Here we tested the hypothesis that OC-STAMP is necessary for the formation of FBGCs derived from murine bone marrow. Our results show that OC-STAMP is significantly expressed in FBGCs, and its inhibition by an anti-OC-STAMP antibody blocks FBGC formation. Because these giant cells are implicated in many clinical conditions, understanding the mechanism of formation, and subsequent function, may provide avenues to inhibit the undesirable effects of FBGCs.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF BONE MARROW MONONUCLEAR CELLS

Six-week old C57/BL6 mice were obtained from the Animal Resources Centre (Perth, Australia). Mice were euthanized at the age of 6–8 weeks and dissected to obtain humerus and tibia. All cytokines were purchased directly from PeproTech Asia (Rehovot, Israel). Cell culture media were obtained from Invitrogen (USA). All experiments with cell culture were in α-MEM media augmented with 10% fetal bovine serum and 1% penicillin–streptomycin antibiotic mix and conducted in 5% CO_2 atmosphere at 37°C. Bone marrow mononuclear cells (BMM) were flushed from bone with medium and then filtered through a 600 μM cell strainer (Invitrogen). Cells were seeded in 150 mm × 25 mm cell culture dishes at a density of $1.5 \times 10^4/cm^2$ augmented with 30 ng/ml of MCSF. After 2 days, adherent cells were separated from the non-adherent cells and plated in 96 and 6-well plates at a density of $1.5 \times 10^4/cm^2$. BMM cultures were differentiated into cell types using cytokines. Macrophage-like cells were generated by adding MCSF (30 ng/ml) to media. Osteoclast-like cells were generated by adding MCSF (30 ng/ml) and RANKL (35 ng/ml) to media. FBGC-like cells were generated by adding GMCSF and IL-4 (both at 50 ng/ml) to media. After fixation with 10% formalin, one batch of cells was TRAP stained (Sigma–Aldrich, USA), and the other was stained with DAPI and phalloidin according to manufacturer's instructions (Molecular Probes, USA). To measure the bone resorption activity of OCs and FBGCs under various treatment conditions, cells were also plated on dentin slices. For inhibition studies, anti-OC-STAMP antibody was added (1:200), and normal rabbit sera were used as a control. Approval was obtained from Griffith University Animal Ethics Committee for all animal procedures.

qPCR ANALYSIS OF OC-STAMP

Cells were seeded at a density of $1.5 \times 10^4/cm^2$ and treated with the appropriate mix of cytokines to establish macrophages, OCs, and FBGCs. The Nucleospin RNA II (Macherey-Nagel, PA, USA) kit was used to extract RNA from cultured cells on each day from Day 0 to Day 4 following the manufacturer's instructions. RNA was converted into cDNA using ImProm-II Reverse Transcriptase (RT, Promega) and oligo dT primer according to the manufacturer's protocols. The following primers were used for qPCR: for OC-STAMP forward primer, TGGGCCTCCATATGACCTCGAGTAG and reverse primer TCAAAGGCTTGTAAATTGGAGGAGT; for 18s positive control forward primer, CTTAGAGGGACAAGTGGCG and reverse primer ACGCTGAGCCAGTCAGTGTA. Expression analysis was performed with Biorad SYBR Green supermix in a Bio-Rad iCycler according to the manufacturer's instructions.

WESTERN BLOT

Macrophages, OCs, and FBGCs were washed twice with PBS and lysed in RIPA buffer (Thermo Scientific, Sydney). Protein concentrations were estimated by the DC protein assay (Biorad) using BSA standards. Equal amount of proteins were separated on SDS–PAGE gels before transfer to polyvinylidene fluoride membranes. Membranes were then blocked with 5% skimmed milk for 1 h and subsequently probed with primary rabbit anti-mouse OC-STAMP or rabbit anti-mouse calnexin antibody (eBiosciences) overnight at 1:500 dilutions. The membranes were then washed five times with tris-buffered saline with 0.5% Tween 20 (TBST) and probed with secondary horseradish peroxidase-conjugated antibody for 1 h. Finally, the membrane was washed five times with TBST buffer and detected using Bio Rad chemiluminescence assay according to the manufacturer's instructions.

CELL COUNT AND STATISTICAL ANALYSIS

The culture treatments were done in triplicate and the entire experiment was repeated six times on different occasions, using six different bone marrow donor mice, with mRNA taken from all experiments. Zero time was used as baseline. Cell count was assessed on the first three independent experiments. After cells were fixed and stained, six non-overlapping images were taken for each of triplicate culture wells. In each image the total number of TRAP-positive multinucleated giant cells (MNGCs) was counted, followed by the number of nuclei in each giant cell and finally the maximum diameter of each MNGC was measured in pixels by the way of Feret's diameter of irregular bodies. Cell count, nuclei count, and size data were established by two independent assessors using the same images and the mean of all data presented. Analysis of variance (ANOVA) was used to compare the means of cell number, nuclei

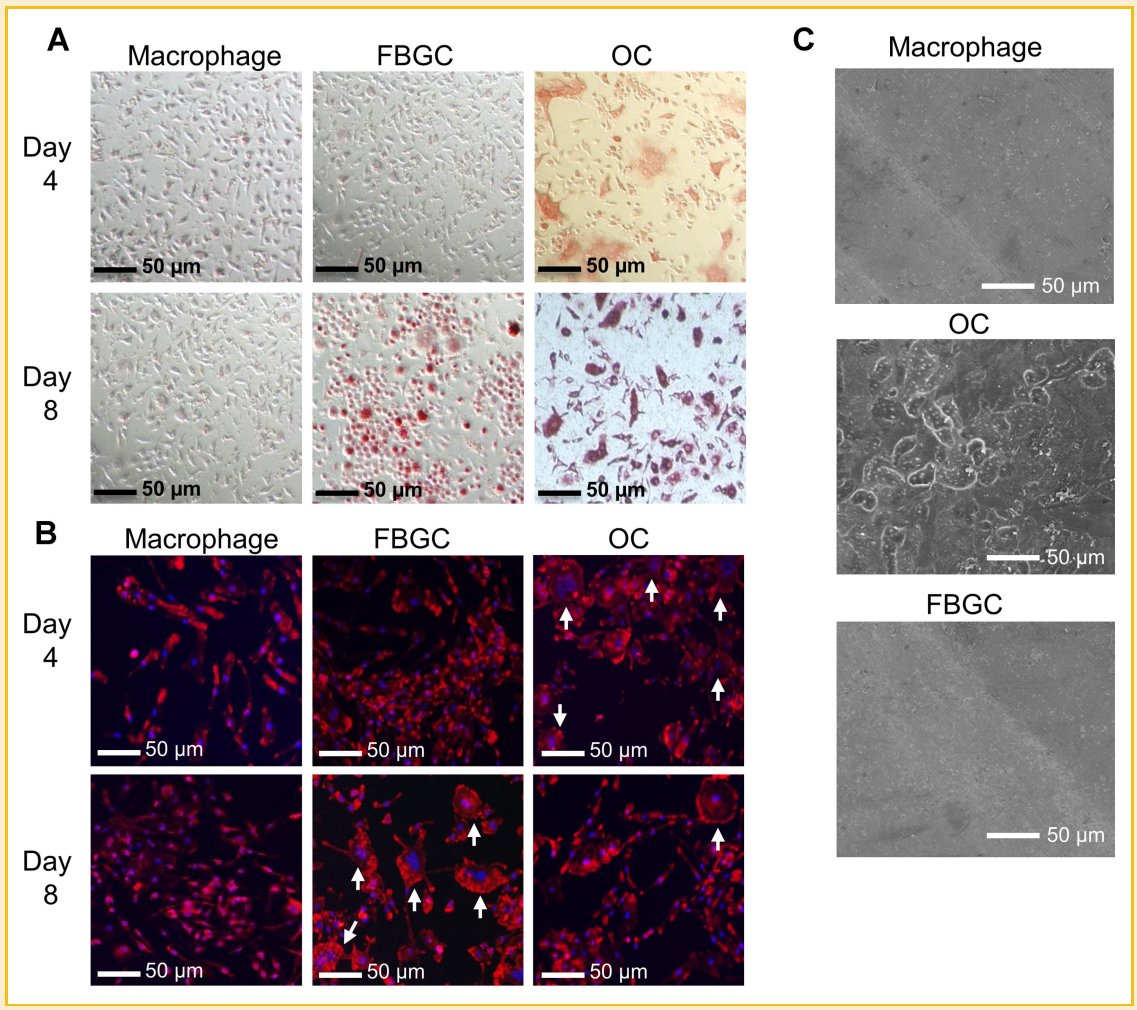

Fig. 1. Characteristics of multinucleated giant cells (MGCs). A: TRAP staining of cells after 4 and 8 days of culture. Macrophages and FBGCs were TRAP-negative on Day 4, but osteoclasts were TRAP-positive. By Day 8, both FBGCs and osteoclasts were TRAP-positive. B: Arrows represent F-actin belt formation in MGCs. The timing of F-actin ring formation was similar to that for TRAP staining. C: Bone resorption assay. Osteoclasts showed typical bone resorption, while macrophages and FBGCs did not resorb bone.

count, and Feret's diameter in different treatment groups. Data and graphs are reported as the effect derived from all experiments combined, as mean ± standard error of mean (SEM). Representative random images are presented in figures to illustrate cell appearance.

RESULTS

CHARACTERIZATION OF FBGCs DERIVED FROM MOUSE BONE MARROW

We first developed a similar staining assay for OCs and FBGCs. When bone marrow monocytes, treated with GMCSF and IL4, were cultured for 4 days, they remained TRAP negative (Fig. 1A). However, when the culture was extended to 8 days, FBGCs were strongly TRAP positive (Fig. 1A). In addition, on Day 8 FBGCs developed the podosome belt structure (Fig. 1B), characteristically found in OCs grown on plastic culture plates [Jurdic et al., 2006].

Although FBGCs generated by GMCSF and IL4 treatment showed characteristic osteoclast markers of podosome belt formation and TRAP expression, such cells were incapable of bone resorption on dentine slices (Fig. 1C).

OC-STAMP IS INDUCED IN FBGCs AND OCs

OC-STAMP expression was significantly higher in FBGCs compared to macrophages (Fig. 2A). Furthermore, OCs exhibited reduction in OC-STAMP expression on Day 4, compared to Day 3, but FBGCs showed a continued increase in OC-STAMP expression (Fig. 2A). Expression at the protein level was confirmed with Western blotting. It was observed that mononuclear cells from bone marrow did not express OC-STAMP, but OCs and FBGCs were both positive for OC-STAMP (Fig. 2B). FBGCs and OCs expressed markedly higher DC-STAMP mRNA compared to macrophages on Day 4, but the difference between OCs and FBGCs was not significant (Fig. 2C). OCs were TRAP positive on Day 4, which is supported at the mRNA level (Fig. 2D).

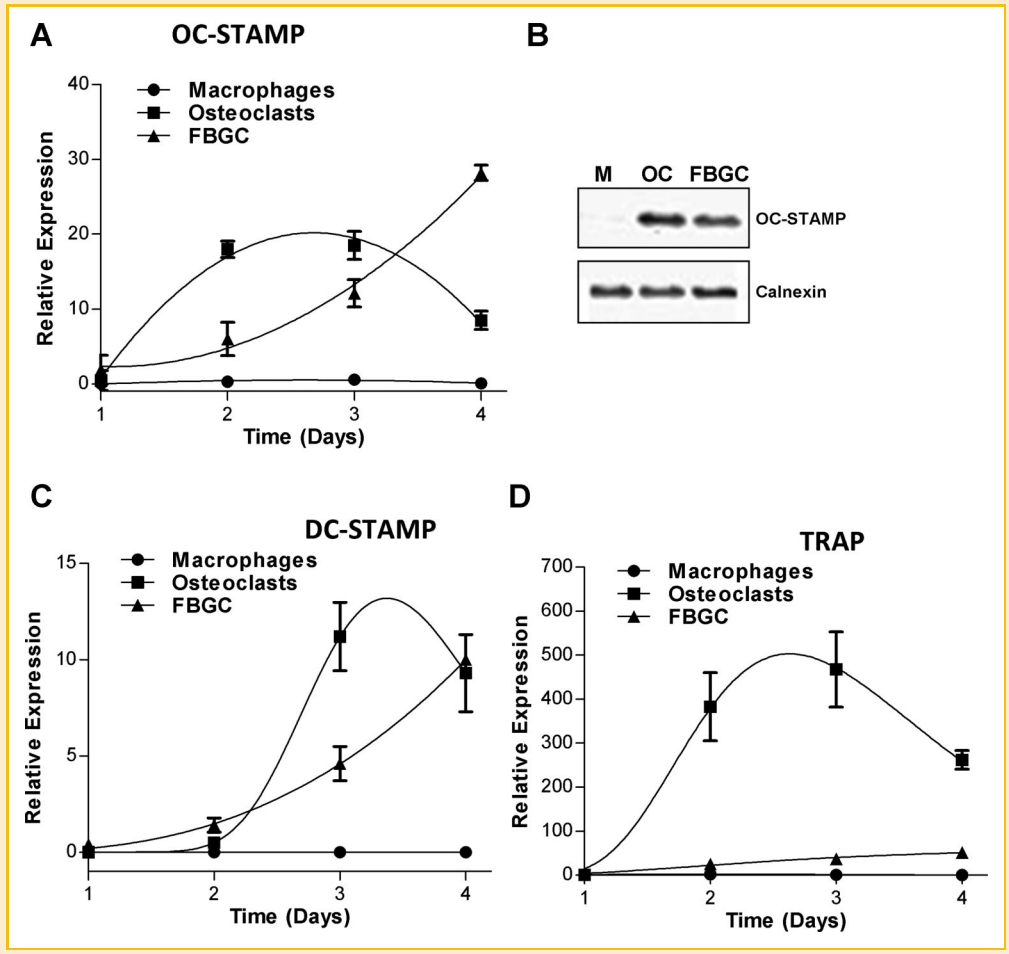

Fig. 2. Induction of OC-STAMP during osteoclast and FBGC formation. A: Osteoclasts and FBGC expressed more OC-STAMP than macrophages ($P < 0.05$). On Day 4, FBGCs expressed OC-STAMP threefold higher than osteoclasts ($P < 0.05$). Mean ± SEM. B: Western blotting confirmed that osteoclasts (OC) and FBGC both express OC-STAMP protein whereas macrophages (M) do not express detectable OC-STAMP. Cultures were at harvested at Day 4. C: DC-STAMP was expressed more highly by osteoclasts compared to FBGC at Day 3, but similarly at Day 4. Both osteoclasts and FBGCs expressed significantly more DC-STAMP than macrophages ($P < 0.05$). D: TRAP was induced in FBGCs relative to macrophages ($P < 0.05$) although this was small in comparison to the high induction of TRAP in osteoclasts during this time interval.

OC-STAMP INHIBITION HINDERS FBGC AND OSTEOCLAST FORMATION

Having established the expression level of OC-STAMP in FBGCs we sought to ascertain its functional role in the formation of FBGCs. When OC-STAMP was inhibited by anti OC-STAMP antibody, a significant reduction in the number of MNGCs was measured for both OCs and FBGCs (Fig. 3A). To eliminate the possibility that a reduction in the number of MNGCs was a result of increased fusion, we counted the number of nuclei in each cell, and also measured the size of multinucleated TRAP-positive cells (Fig. 3B). Both the number of nuclei (Fig. 3C) and size of the cells were reduced following inhibition of OC-STAMP (Fig. 3D). Anti OC-STAMP antibody did not affect the proliferation of BMMs; reflected in the number of nuclei in both the OC-STAMP inhibited and control cultures. There was no significant difference between the numbers of nuclei per field in treated or control cells (Fig. 4). When these cells were plated on dentin slices, OC-STAMP inhibition also resulted in the absence of bone resorption (Fig. 5). Finally, OCs and FBGCs failed to form the characteristic podosome belt when treated with anti-OC-STAMP antibody (Fig. 6).

DISCUSSION

Fusion of macrophages leads to the formation of FBGC and OCs. Both of these cells are located in close proximity during peri-implantitis [Konttinen et al., 2006]. FBGCs observed near rejected implants are reported to be TRAP-positive [Anazawa et al., 2004]. Conversely, FBGCs that are generated during culture, in vitro, are TRAP-negative [Moreno et al., 2007]. We have shown that FBGCs grown for 4 days remain TRAP negative, but when the culture period was extended to 8 days, FBGCs became positive for TRAP staining. TRAP is an important marker of osteoclast activity [Keiler et al., 2012], and OCs from mice lacking TRAP staining were dysfunctional, resulting in an osteopetrotic phenotype [Hayman et al., 1996]. IL-4, involved in the induction of FBGCs, suppresses RANKL-

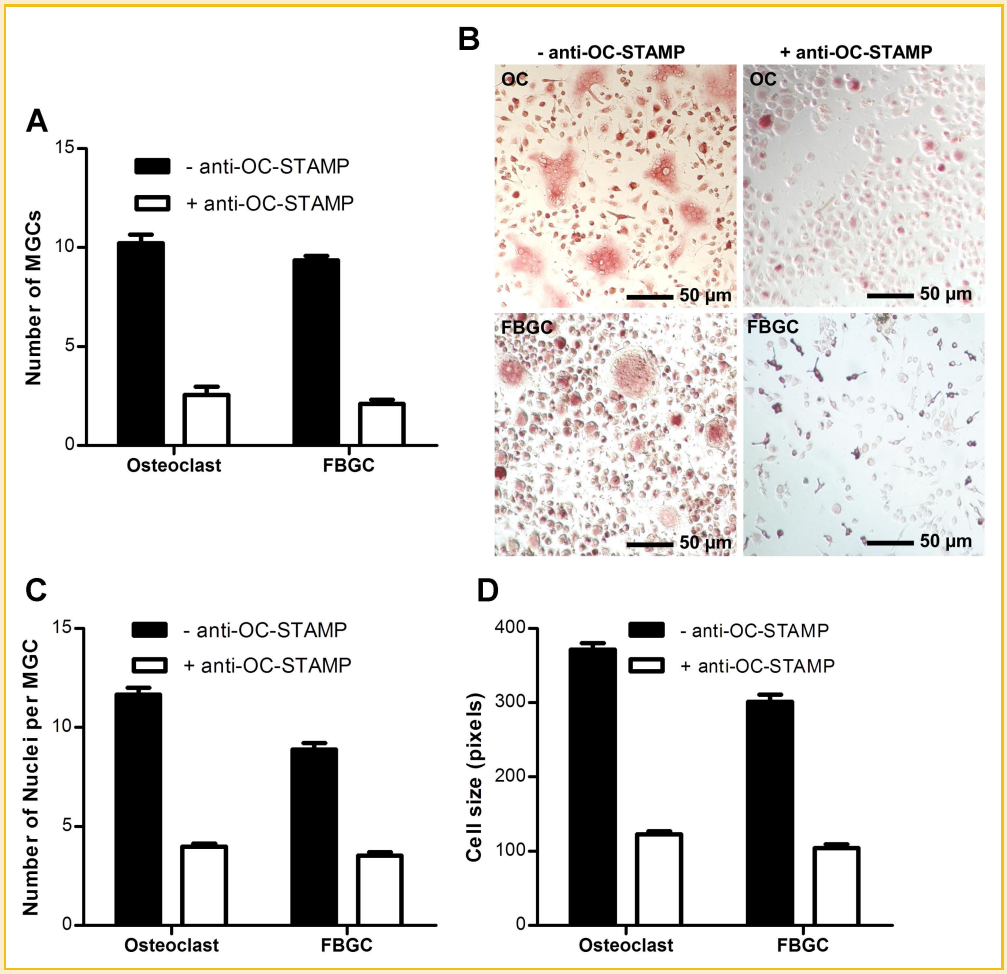

Fig. 3. Inhibition of OC-STAMP resulted in a significant reduction of fusion in both osteoclast (OC) and FBGCs. A: There is a significant reduction in the number of multinucleated giant cells (MGCs) in osteoclast cultures treated with anti-OC-STAMP antibody. The same effect can be seen in FBGC cultures. B: Effect of anti-OC-STAMP antibody on multinucleation and TRAP staining in cultures of OC and FBGC. OC (upper two images) can be seen as multinucleated TRAP positive giant cells while anti-OC-STAMP antibody significantly inhibited the formation of OC. FBGCs (lower left images) can be seen as multinucleated giant cells, while OC-STAMP antibody prevented the formation of FBGCs (lower right). C: Anti-OC-STAMP antibody also reduced the number of nuclei in those multinuclear cells that did form in both the FBGC and osteoclast cultures. D: Anti-OC-STAMP antibody treatment resulted in a reduction in the size of osteoclasts and FBGCs that did form. Error bars in A, C, and D represent SEM. Data in graphs A, C, and D are derived from two observers measuring the same images independently as described in the Materials and Methods Section, assessing the following number of MGCs for each treatment: 552 (OC), 138 (OC plus anti-OC-STAMP antibody), 505 (FBGC), and 114 (FBGC plus anti-OC-STAMP antibody).

mediated TRAP expression, but also increases TRAP expression in the absence of RANKL [Yu et al., 2009]. Furthermore, IL-4 is considered a switch that directs bone marrow cells toward FBGCs rather than OCs [Bendixen et al., 2001; Moreno et al., 2007]. Moreover, these FBGCs survive for longer periods of time [Anderson, 1988], compared to OCs. [Akiyama et al., 2005]. In the 4-day interval shown in Figure 2D, TRAP mRNA was low in FBGC relative to OCs (but still 20-fold induced relative to macrophages) and TRAP activity accumulates so that cells are positive for TRAP stain at 8 days. Our data assists in explaining the difference between in vitro (TRAP negative) and in vivo observations of FBGC (generally TRAP positive). A reasonable, simple explanation for this difference relates to cell age: probably FBGC are rather old when observed in vivo (at least older than 8 days) as FBGC exist in chronic, not acute, inflammatory conditions.

During osteoclast differentiation, the cytoskeletal structure is organized to facilitate adhesion of the osteoclast to bone surfaces. This includes the development of podosomes, which are key structures involved in macrophage fusion to form FBGCs, and their subsequent function [DeFife et al., 1999]. Podosomes found in FBGCs are similar to those in OCs, and provide a confined environment for the function of both of these cells. Furthermore, F-actin is consistently observed in OCs and leads to cytoskeletal changes such as F-actin ring formation. These structures play a significant role in cell integrity, shape, motility, and adhesion [Akisaka et al., 2001]. The F-actin is a double helical polymer, derived from a monomer of actin in a globular fashion, to which phalloidin binds specifically [Wulf et al., 1979]. We hypothesized that the podosomes found in FBGCs would arrange in the same ring structure as OCs. This was confirmed using phalloidin staining.

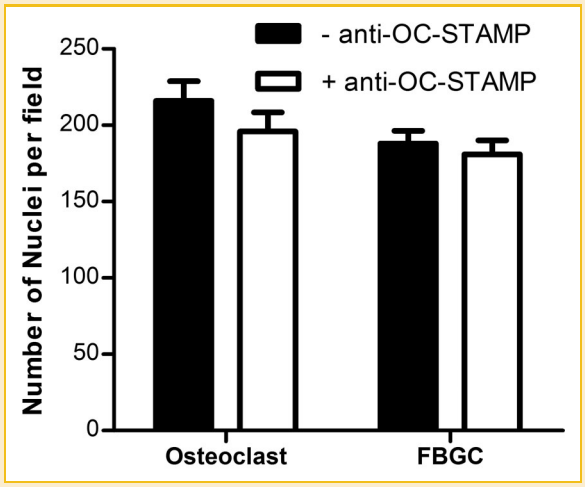

Fig. 4. OC-STAMP inhibition has no effect on total nuclear count. There is no significant difference in the number of nuclei per field of view in anti-OC-STAMP antibody treated versus control group for both osteoclast and FBGC cultures. Mean ± SEM.

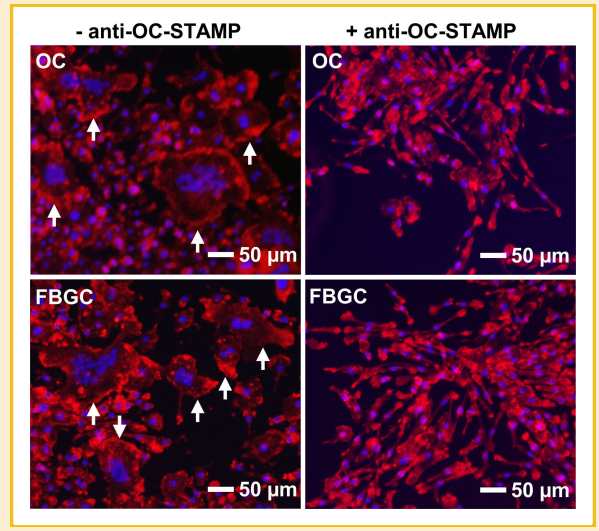

Fig. 6. Arrows represent podosome belt formation that is inhibited by anti-OC-STAMP antibody as seen in DAPI (blue) and phalloidin staining for F-actin (red). Images on the left shows characteristic osteoclast (OC) and FBGCs with multiple nuclei and podosome belts, while the images on the right shows inhibition of podosome belt formation in both cell types.

OC-STAMP is identified as a protein, induced by RANKL during osteoclast formation [Yang et al., 2008]. Knock-down of OC-STAMP resulted in significant reduction in the expression of RANK and c-Fms [Kim et al., 2011], the receptors for RANKL and MCSF, respectively (both important factors for osteoclast formation) [Hattersley et al., 1991; Dougall et al., 1999]. DC-STAMP is a cell surface receptor that is important for osteoclastogenesis and FBGC formation [Kukita et al., 2004; Yagi et al., 2005]. Furthermore, OC-STAMP shares a 193-residue region of amino acid similarity to a DC-STAMP family consensus; although overall homology between OC-STAMP and DC-STAMP is only 17% [Yang et al., 2008]. Since OC-STAMP has a seven transmembrane pass receptor structure, similar to DC-STAMP (and DC-STAMP is essential for osteoclast and FBGCs formation), we examined OC-STAMP expression in OCs and FBGCs. We found that FBGCs had significantly higher expression of OC-STAMP ($P < 0.05$). We therefore conclude that GMCSF and IL-4 induce OC-STAMP expression during the formation of FBGCs.

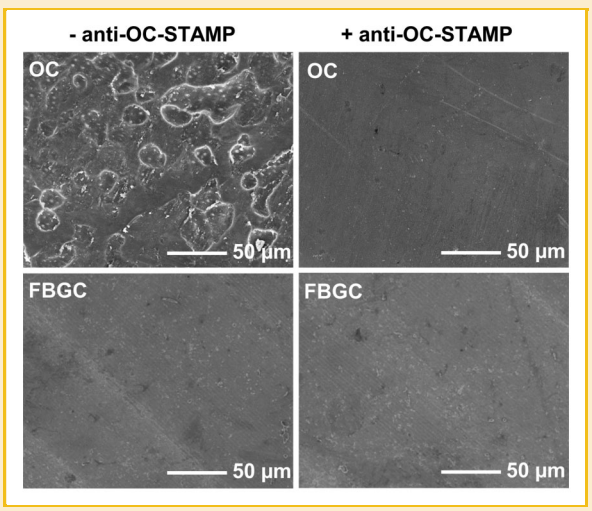

Fig. 5. The bone resorption capacity of osteoclasts (OC) on dentin was eliminated when OC-STAMP was inhibited using anti-OC-STAMP antibody (upper two panels). Scanning electron microscopy image on the top left shows bone resorption pits caused by osteoclast cultures and top right shows absence of pits on dentin in osteoclast cultures treated with anti-OC-STAMP antibody. In contrast, FBGC cultures (bottom left) under either condition fail to form pits on dentin.

During the formation of multinucleated OCs, it is known that OC-STAMP expression is induced by RANKL, and that inhibition by antibodies or siRNAs blocks osteoclast formation [Yang et al., 2008]. Furthermore, OC-STAMP knockout mice exhibit a complete lack of cell–cell fusion in FBGCs and OCs [Miyamoto et al., 2012], which is consistent with our results. Knock-down of OC-STAMP resulted in decreased macrophage fusion, and anti-OC-STAMP antibody resulted in the inhibition of bone resorption activity of OCs [Kim et al., 2011].

In conclusion, OCs and FBGCs showed increased expression of OC-STAMP compared to macrophages. Inhibition of OC-STAMP resulted in decreased fusion during the formation of OCs and FBGCs. Most importantly GMCSF and IL-4 induced the expression of OC-STAMP in FBGCs. Inhibition of OC-STAMP by monoclonal antibodies could provide a therapeutic avenue for the control of FBGCs at sites of prostheses, implants, and medical devices.

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