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Caspase-2 modulates osteoclastogenesis through down-regulating oxidative stress

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Abstract

The loss of caspase-2 (Casp-2) in mice results in an osteopenic phenotype associated with increased numbers of osteoclasts in vivo. In this study, we show that Casp-2 is involved in osteoclastogenesis. Protein levels of Casp-2 decrease during the differentiation of macrophages to osteoclasts. Furthermore, siRNA-mediated Casp-2 knockdown in osteoclast precursors or differentiation of bone marrow macrophage (BMM) precursors from *Casp2*$^{-/-}$ mice results in increased osteoclast numbers and tartrate-resistant acid phosphatase (TRAP) activity. *Casp2*$^{-/-}$ osteoclasts are larger in size compared to wild-type osteoclasts and exhibited increased numbers of nuclei, perhaps due to increased precursor fusion. The loss of Casp-2 did not alter earlier stages of differentiation, but had a greater consequence on later stages involving NFATc1 auto-amplification and pre-osteoclast fusion. We have previously shown that the loss of Casp-2 results in increased oxidative stress in the bone. Reactive oxygen species (ROS) is known to play a critical role in late osteoclast differentiation and we show that total ROS and specifically, mitochondrial ROS, significantly increased in *Casp2*$^{-/-}$ BMM precursors after RANKL administration, with a concomitant reduction in FoxO3a and its target antioxidant enzymes, catalase and superoxide 2 (SOD2). Because mitochondrial ROS has been identified as a putative regulator of the later stages of differentiation, the heightened ROS levels in *Casp2*$^{-/-}$ cells likely promote precursor fusion and increased osteoclast numbers. In conclusion, our results indicate a novel role of Casp-2 in the osteoclast as a modulator of total and mitochondrial ROS and osteoclast differentiation.

Keywords

Caspase-2; Osteoclastogenesis; Oxidative stress

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Introduction

Bone homeostasis is dependent upon a balance between bone resorption by osteoclasts and bone formation by osteoblasts. In conditions where this balance is not maintained, pathological disorders such as osteoporosis are manifested. One of the major underlying factors that have been shown to promote excess bone resorption and lead to the development of bone loss is oxidative stress. For example, oxidative stress was shown to have a significant negative correlation with bone mineral density (BMD) in the lumbar vertebrae and femoral neck in patients with postmenopausal osteoporosis [1]. In addition, several studies have shown that postmenopausal osteoporosis in humans as well as rodent models is associated with decreased antioxidant defenses, which subsequently leads to higher levels of reactive oxygen species (ROS) and bone loss [2–4].

Oxidative stress occurs when there are increased levels of ROS, such as hydrogen peroxide and superoxide, resulting in macromolecular damage within the cell. Interestingly, ROS has been shown to be an instrumental component in promoting osteoclast differentiation and activity [5–8]. In response to receptor activator of nuclear factor kappa-B ligand (RANKL), ROS levels increase and activate signaling pathways such as mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinases (JNKs), extracellular signal-related kinases (ERKs), and p38, IKBα, and nuclear factor kappa B (NF-κB), culminating in the activation of the master osteoclast transcriptional regulator, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) [5,6,9]. Also, the activation of Ca^{2+}-calmodulin and other proteins result in NFATc1 activation and osteoclast gene transcription [10,11]. The initial rise in Ca^{2+} levels also appears to be dependent upon ROS [12]. During this early differentiation stage, the primary focus is on BMM proliferation and maintaining pro-osteoclastogenesis signaling. Later, the cell enters a late-differentiation stage marked by the auto-amplification of NFATc1 driven by long lasting Ca^{2+} oscillations that greatly increases osteoclast gene transcription and drives committed osteoclast precursors towards fusion [9,12]. Concurrently, ROS levels also rise, although the identity of the particular species and its source has not been fully elucidated. One possibility is the mitochondria, particularly because increased mitochondrial biogenesis occurs during this point in differentiation [13,14]. In addition, mitochondria are the highest producers of ROS through the electron transport chain, and would likely be primary contributors of ROS during osteoclastogenesis. Depletion of mitochondrial ROS levels leads to a decrease in osteoclast numbers [15,16]. However, downstream mediators of ROS in osteoclast differentiation remain unclear. We posit Casp-2 as a candidate regulatory molecule involved in this process.

Casp-2 is a cysteine aspartate protease that is well known for its role during cellular apoptosis [17–20]. More recent studies have indicated, though, that Casp-2 may be involved in a wider variety of cellular process including the regulation of antioxidant enzymes. Studies by Shalini et al. show that mouse embryonic fibroblasts (MEFs) from *Casp2*$^{-/-}$ mice exhibited higher levels of ROS compared to wild-type (WT) [21]. Furthermore, in cells knocked down for *Casp2*, levels of the antioxidant enzymes superoxide dismutase 2 (SOD2), catalase, and glutathione peroxidase (GSH-Px) were decreased as well as the upstream transcriptional regulator, FoxO3a. Importantly, overexpression of Casp-2 in this model resulted in increased levels of FoxO3a as well as the downstream antioxidant SOD2

and GSH-Px, establishing a link between Casp-2 and FoxO3a expression. The loss of Casp-2 has been shown to lead to a premature aging phenotype [21,22], which includes significantly decreased BMD compared to age-matched WT mice. The decrease in BMD in old mice (>24 mos) was accompanied by decreased bone volume, increased urinary deoxypyridinoline (DPD), and increased numbers of osteoclasts, suggesting that the bone loss phenotype in *Casp2*$^{-/-}$ mice could be osteoclast-based [22]. The observed in vivo increase in osteoclast numbers may be explained by decreased osteoclast apoptosis and/or increased osteoclast differentiation. We have recently addressed the contribution of Casp-2 in osteoclast apoptosis wherein we showed that the loss of Casp-2 attenuated osteoclast apoptosis in response to oxidative stressors [23]. To address the underlying mechanistic role of Casp-2 in osteoclastogenesis and its modulation of and by ROS, we ablated Casp-2 in bone marrow macrophages and osteoclasts using either gene knockdown or used precursors derived from a mouse model with a global *Casp2* deletion. In both cases, we show that deletion of Casp-2 augments osteoclastogenesis that correlates with an increase in oxidative stress. Interestingly, Casp-2 affects the later stage of osteoclast differentiation involving cell fusion.

Materials and methods

Mice

Casp2$^{-/-}$ mice were originally generated by Dr. Junying Yuan of Harvard University and kindly provided by Dr. Carol Troy of Columbia University with Dr. Yuan's consent. The mice were backcrossed with C57Bl/6 for ten generations. All mice were housed in micro-isolator-topped cages and maintained in a pathogen-free environment at the AAALAC-accredited UTHSCSA animal facility following the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. We used male WT and *Casp2*$^{-/-}$ mice aged 3–6 weeks for these studies.

Cell culture

All cells were grown in Gibco αMEM (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Biosera, Kansas City, MO) and antibiotics. Primary bone marrow was isolated according to standard protocols. In brief, mice were sedated with isofluorane (Baxter, Deerfield, IL) and sacrificed by cervical dislocation. Bone marrow cells were collected by washing the marrow cavity αMEM delivered via a 21 G needle. Cells were cultured overnight and the non-adherent fraction was separated and allowed to expand for 3 days in 30 ng/mL CSF-1 (R&D Systems, Minneapolis, MN). BMMs were harvested and plated with 30 ng/mL CSF-1 and 10 ng/mL RANKL (R&D Systems) for differentiation into osteoclasts.

Cell transfections with siRNA

RAW 264.7 cells were plated and allowed to grow for 24 h. Cells were then transfected in OPTIMEM containing Lipofectamine® RNAiMAX (Life Technologies) and 33 nM siRNA duplexes, following the manufacturer's protocols. Casp-2 siRNA (Qiagen, Valencia, CA; MM_Casp2_3 SI00941717 5′-CAGGGTCACTTGGAAGACTTA-3′) or AllStars Negative Control (Qiagen) was used. After 1 h, RANKL was added to each well. Cells were

allowed to differentiate for 2 days and were either scraped in RIPA buffer (Santa Cruz Biotechnology, Dallas, TX) or were given fresh media containing siRNA duplexes and RANKL. At day 4 cells were scraped again or were used for TRAP staining or TRAP activity assays.

TRAP (tartrate-resistant acid phosphatase) staining and activity assays

TRAP staining was accomplished with a Leukocyte Acid Phosphatase Staining Kit (Sigma. St. Louis, MO) according to the manufacturer's protocols. Total numbers of $TRAP^+$, multi-nucleated (>3) cells (MNCs) per well of a 48-well plate were counted. In addition, images of these wells were used to assess up to 10 osteoclasts for nuclear number and cell size with ImageJ software and numbers of nuclei/unit area were calculated. A total of 100 osteoclasts were assessed per group. Osteoclasts in these images were also categorized according to number of nuclei and compared among groups. To measure TRAP activity, live cells were incubated for 30 min at 37 °C in a pre-warmed solution containing 4% acetate, 4% tartrate, 0.1% sodium dodecyl sulfate, and 6 tablets of SIGMA FAST™ p-nitrophenyl phosphate (Sigma). The resulting supernatant was transferred to a fresh 96 well plate and read at 405 nm on a BioRAD iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Experiments were performed in triplicate with at least three replicates per experiment.

Western blotting

Cells at different stages of differentiation were lysed with RIPA buffer (Santa Cruz Biotechnology, Dallas, TX). Protein concentration was assessed with a Micro BCA protein assay kit (Thermo Scientific, Waltham, MA) and 30 μg protein was loaded per lane on 10% SDS-PAGE gels. Following transfer, nitrocellulose membranes were blocked with either 5% nonfat dry milk or 5% BSA and incubated with primary antibody overnight at 4 °C. Primary antibodies were used to detect Casp-2 (Millipore, Billerica, MA), GAPDH (Life Technologies), cathepsin K (CtsK; Abcam, Cambridge, MA), NFATc1 (Santa Cruz Biotechnology), catalase (Santa Cruz Biotechnology), SOD2 (Santa Cruz Biotechnology), FoxO3a (Cell Signaling Technology, Danvers, MA), and MAPK (total and phosphorylated Akt, IκBα, ERK, p38, and JNK; Cell Signaling Technology). Secondary antibodies compatible with the LI-COR infrared imaging system were used that included anti-rat, mouse, or rabbit IRDye® 800CW or IRDye® 680RD (LI-COR, Lincoln, NE). Blots were imaged on a LI-COR Odyssey system and assessed by densitometry using in Image Studio Lite V3.1 software. Results were normalized to the GAPDH and in some cases also to the day 0 (macrophage/M0 or BMM) samples. Experiments were performed at least in triplicate.

Immunocytochemistry

Primary BMMs and osteoclasts were cultured in 24-well plates on glass coverslips pretreated with 4.7% HCl. Cells were first incubated with mouse IgG to block non-specific binding, rinsed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 15 min at room temperature. After treating with blocking solution (2% goat serum, 2% fish skin gelatin, 0.25% Triton X-100 and 1% bovine serum albumin in PBS) for 30 min, cells were labeled with rabbit anti-caspase 2 antibody (Abcam), followed by rhodamine-conjugated goat anti-rabbit antibody and phalloidin-Alexa488 (Invitrogen).

Nuclei were labeled with DAPI (Life Technologies). Cells were imaged on an Olympus BH-2 fluorescence microscope and the images were processed offline with NIH ImageJ software.

ROS detection

Macrophages were plated into 96-well tissue culture plates and incubated with 30 ng/mL CSF-1 alone or with 30 ng/mL CSF-1 and 10 ng/mL RANKL to induce osteoclast differentiation. At the start of the experiment, cells were washed with warm PBS to remove the culture medium. Cells were then incubated in a dye solution containing either 10 μM CM-H_2DCFDA (Life Technologies) for total ROS measurements or 5 μM MitoSOX Red (Life Technologies) for mitochondrial superoxide detection, respectively. Cells were then washed twice with PBS and the fluorescence was measured with a BioTek Synergy HT at 485 ex/528 em (DCF-DA) and 530 ex/590 em (MitoSOX). Experiments were performed in triplicate with at least 6 replicates per experiment.

Statistics

Statistical analysis was performed using GraphPad Prism 5 (GraphPad, San Diego, CA) with data expressed as mean ± standard error of the mean (SEM). Statistical analyses included the Student's unpaired t-test to compare two groups and one-way ANOVA with the Tukey post-test for analysis of multiple groups. Asterisks indicate the degree of significant differences compared with the controls (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). n value refers to the numbers of separate experiments.

Results

Casp-2 expression decreases during osteoclastogenesis

Although the loss of Casp-2 in global knockout mouse model has been shown to lead to enhanced bone resorption in old mice [22], the role of Casp-2 in differentiating macrophages has not yet been explored. Therefore, we first determined if Casp-2 is present in BMMs and in differentiated osteoclasts via immunoblotting. Both RAW 264.7 macrophage cell line and primary BMMs from WT mice were stimulated with RANKL to induce osteoclastogenesis. The immunoblotting results showed the expression of Casp-2 in RAW 264.7 cells (Fig. 1A) and in primary BMMs (Fig. 1B). Casp-2 protein levels remained relatively unchanged during the first 2 days of differentiation. However, at day 3, Casp-2 levels dropped by approximately 50% as compared to its expression in the BMM, day 1 and day 2 samples. During the last day of culture, which typically contains the maximum numbers of osteoclasts, the Casp-2 protein level declined further in RAW 264.7 and primary cells. These data suggest that the level of Casp-2 inversely correlated with osteoclast differentiation.

Reduction in Casp-2 levels results in increased osteoclast numbers

In order to determine whether this is due to a direct effect on osteoclast differentiation, siRNA was used to knock down Casp-2 in RAW 264.7 macrophages prior to RANKL addition and then again on day 2 (Fig. 2). Protein samples were collected at days 2 and 4. *Casp2* knockdown resulted in ~50% reduction in protein levels in pre-osteoclasts and osteoclasts at days 2 and 4, respectively (Fig. 2A, densitometry in right panel). Casp-2

reduction led to a significant increase in osteoclast numbers (Fig. 2B) and in increased TRAP activity at day 4 (Fig. 2C). As further confirmation of increased osteoclasts, CtsK protein levels were significantly increased in cultures treated with Casp-2 siRNA (Fig. 2E). However, when NFATc1 levels were evaluated in both day 2 pre-osteoclast and day 4 osteoclast cultures, there were trends of increase, but no significant differences compared to the control (Fig. 2D). These data suggest that Casp-2 may have a greater impact during the late stage of differentiation. Therefore, in a separate experiment, Casp-2 siRNA was administered only at day 2 post-RANKL addition rather than in precursors. As shown in Figs. 2F and G, application of siRNA at day 2 of differentiation was sufficient to promote osteoclastogenesis with both increased osteoclast numbers as well as TRAP activity.

To further support the results from siRNA knockdown of Casp-2, primary osteoclasts were differentiated from BMMs derived from WT or *Casp2*$^{-/-}$ mice. Immunofluorescence studies showed that BMMs and osteoclasts from WT animals stained positive for Casp-2 (Fig. 3). The staining pattern of Casp-2 staining is similar to previously reported study [20]. This staining is specific since the signals were greatly diminished in cells isolated from Casp-2 knockout mice. The some “ring-like” staining co-localized with phalloidin also shown in cells depleted of Casp-2 is most likely an artifact due to bleed-through fluorescence from the phalloidin staining. Immunoblotting data further confirmed a complete lack of Casp-2 protein at all points during the differentiation process in *Casp2*$^{-/-}$ cells (Fig. 4A). Osteoclasts derived from *Casp2*$^{-/-}$ mice appeared to be greater in numbers and larger in size compared to those from WT animals (Fig. 4B). Total numbers of osteoclasts were counted in cultures derived from *Casp2*$^{-/-}$ or WT mice, to reveal a 1.5-fold increase in osteoclast numbers in the absence of Casp-2 (Fig. 4C). Next 100 osteoclasts from each well were assessed for both osteoclast area and nuclei number, and the number of nuclei per cell area was calculated. Osteoclasts derived from *Casp2*$^{-/-}$ mice contained more nuclei on average than WT control (Fig. 4D). Furthermore, osteoclasts and other TRAP^{+} precursor cells in the final culture were categorized by total number of nuclei. In *Casp2*$^{-/-}$ cultures, there were significantly less numbers of cells containing 1–2 nuclei, suggesting that overall cell fusion was increased (Fig. 4E). Although total numbers of osteoclasts were increased in *Casp2*$^{-/-}$ cultures, only larger osteoclasts with 11 and greater nuclei were significantly increased compared to the control. Next, CtsK protein levels and TRAP activity were assessed in both *Casp2*$^{-/-}$ and WT osteoclast cultures as further evidence of increased numbers of osteoclasts. Similar to cultures knocked down for *Casp2*, the complete loss of Casp-2 led to increased TRAP activity (Fig. 5A) as well as higher levels of CtsK protein (Fig. 5B) in osteoclasts. The protein levels for the transcription factor NFATc1 were assessed in order to verify that increased osteoclastogenesis was occurring (Fig. 5C). Immunoblots revealed that NFATc1 protein levels were similar between the two groups at day 2 of differentiation as was also seen in the knockdown cultures. However, at day 3 there was a significant increase in NFATc1 levels in the *Casp2*$^{-/-}$ cultures, supporting the hypothesis that the loss of Casp-2 affects late differentiation rather than the early stages.

To evaluate whether the loss of Casp-2 had an effect on early osteoclast differentiation, BMMs isolated from both *Casp2*$^{-/-}$ and WT mice were serum starved for 6 h and then incubated with 100 ng/mL RANKL for 0, 5, 10, 15, and 30 min. As seen in Fig. 6, stimulating BMMs with RANKL led to an increase in the phosphorylation of signaling

proteins involved in the initial induction of osteoclastogenesis signaling including Akt, IκB, and MAPKs (JNK, ERK and p38), which peaked at 10–15 min. If Casp-2 is involved in the early stage of osteoclastogenesis, an increase in the phosphorylation of the early signaling pathways would be expected. However, at no point was there a significant difference in phosphorylated/total protein levels between WT and *Casp2*$^{-/-}$ cells.

The loss of Casp-2 leads to higher levels of total and mitochondrial ROS and correlates to decreased antioxidant levels

Because osteoclastogenesis is known to be dependent on total as well as mitochondrial ROS [5,6,15], we evaluated how the loss of Casp-2 might affect ROS levels in BMMs and osteoclasts. Cells from WT and *Casp2*$^{-/-}$ mice were cultured as previously described and both total ROS and mitochondrial superoxide were measured at each day during the differentiation process. Although there was no significant difference in ROS levels between WT and *Casp2*$^{-/-}$ BMMs, higher total ROS levels were seen during the differentiation process, with a significant increase observed at D1 and D3 in cells from *Casp2*$^{-/-}$ mice (Fig. 7A). Results from mitochondrial ROS detection yielded similar results with significant increases seen at D1, D2, and D4 (Fig. 7B). Throughout the differentiation process, MitoSOX intensity was higher in cells from *Casp2*$^{-/-}$; however, it was only significant at D1, D2, and D4. Interestingly, both total ROS and mitochondrial superoxide peaked at D3 in these studies, coinciding with the increase in NFATc1 seen in Fig. 5C.

Increased ROS in MEFs lacking Casp-2 has been linked to an impaired antioxidant defense system [21]. Specifically, Casp-2 was proposed to regulate FoxO3a that is involved in antioxidant enzyme transcription [21]. Therefore, whether this same mechanism was affecting ROS levels in Casp-2-deficient cells during osteoclastogenesis was examined. Protein samples from WT and *Casp2*$^{-/-}$ BMMs, pre-osteoclasts, and osteoclasts were collected and assessed for FoxO3a, catalase, and SOD2 levels (Fig. 8). Catalase and SOD2 are cytosolic and mitochondrial antioxidants, respectively, that have been shown to be regulated by FoxO3a [24]. In BMMs not treated with RANKL, the loss of Casp-2 had no effect on FoxO3a or SOD2 levels, but there was a significant decrease in catalase compared to WT. However, in pre-osteoclasts and osteoclasts obtained at days 2 and 4 of culture, respectively, *Casp2*$^{-/-}$ cells had significantly decreased protein levels of FoxO3a, catalase, and SOD2. Together, these data suggest that Casp-2 regulates the levels of ROS during osteoclastogenesis. The increased ROS as a result of Casp-2 deletion is a likely mechanism leading to the enhancement of osteoclast differentiation.

Discussion

The loss of Casp-2 in vivo in mice is associated with bone loss that appears to be at least partly due to enhanced osteoclast activity [22,23]. Specifically, increased levels of the urinary bone resorption marker deoxypyridinoline (DPD), and increased numbers of osteoclasts were present in *Casp2*$^{-/-}$ mice compared to age-matched WT mice [22]. In addition, there was also increased skeletal uptake of Technetium-99 m methylene diphosphonate (99mTc-MDP), which is associated with overall increased bone remodeling by osteoclasts [22] and seen clinically in osteoporotic patients [25]. In a more recent paper

regarding bone quality in *Casp2*$^{-/-}$ mice, Sharma et al. further explored the consequence of Casp-2 loss in bones in vivo with micro-CT, DXA, and mechanical strength testing, and concluded that the loss of Casp-2 resulted in weaker bones that could be attributed to increased osteoclast numbers and activity [23]. However, the exact mechanism by which the loss of Casp-2 contributes to the bone loss phenotype is unclear, particularly with regard to whether increased osteoclast numbers are due to a reduction in osteoclast apoptosis and/or increased osteoclast differentiation. We have recently shown that the loss of Casp-2 decreases osteoclast apoptosis [23] and is associated with higher levels of oxidative stress. In this study, we established that Casp-2 protein levels decrease during osteoclast differentiation and plays an important role in regulating later states of osteoclastogenesis. Furthermore, the loss of Casp-2 led to an increase in osteoclastogenesis and higher overall levels of total and mitochondrial ROS, perhaps driven by a reduction in FoxO3a-regulated antioxidant enzymes.

Compared to WT or non-transfected controls, cells lacking Casp-2 were shown to promote increased osteoclast differentiation as indicated by increased osteoclast numbers, TRAP activity, and CtsK protein levels. Furthermore, *Casp2*$^{-/-}$ osteoclasts also had higher numbers of nuclei per cell area suggesting that increased fusion was taking place during the differentiation process. This is correlated by an increase in NFATc1 protein levels at day 3 of differentiation. However, NFATc1 was not significantly higher in *Casp2*$^{-/-}$ cells earlier in the differentiation process, suggesting that the loss of Casp-2 may only impact later stages of differentiation that are characterized by NFATc1 autoamplification and committed precursor fusion. The addition of siRNA directed against *Casp2* delivered at day 2 of differentiation rather than at day 0 was sufficient to induce increased osteoclastogenesis as shown through increased osteoclast numbers as well as TRAP activity in Figs. 2F and G. Therefore, the data suggested that the loss of Casp-2 even at the stage when cells are actively differentiating is sufficient to yield increased osteoclastogenesis.

Casp-2 deficiency could also lead to decreased osteoclast apoptosis [23]. Given that the loss of Casp-2 is involved in promoting cell survival, this may be a possible reason for the dramatic Casp-2 down-regulation that occurs during osteoclastogenesis. Indeed, our study confirmed that Casp-2 levels in the differentiated osteoclasts are quite low compared to the undifferentiated precursors [23]. Importantly, the changes in Casp-2 that were seen involved the proform of the protein rather than the shorter active forms. This argues for a non-apoptotic role of Casp-2 that does not involve cleavage in osteoclast differentiation. Non-apoptotic roles of Casp-2 have been proposed including antioxidant enzyme regulation, autophagy regulation, and tumor suppression [21, 26,27]. The decrease of the preform of caspase-2 is correlated with the differentiation of osteoclast precursors and the maturation of osteoclasts. The timing also matches with the enhanced levels of ROS. Interestingly, a recent paper has shown that FoxO3a and its downstream antioxidant catalase exhibit similar reductions during osteoclastogenesis [16]. Correspondingly, the numbers of osteoclasts are increased after deletion of Casp-2. Interestingly, Casp-2 down-regulation may be a feature in some terminally differentiated cells, particularly neurons [28]. In a report by Pistritto et al. [29], silencing Casp-2 increased the expression of neuronal differentiation markers, indicating that Casp-2 activation may impede differentiation and, instead, lead to cellular apoptosis. However, the regulatory mechanism of Casp-2 down-regulation remains largely

unknown although evidence from down-regulation induced by rottlerin suggests that it is mediated by the proteasome [30]. Osteoclast differentiation is dependent upon ROS, with the highest levels seen during day 2 as reported previously [12]. Therefore, it is possible that a reduction in Casp-2 occurs in response to the increased ROS levels in order to prevent apoptosis initiation and to initiate osteoclast differentiation.

In the past few decades, the crucial role of ROS in the osteoclast differentiation has established [5,6,16]. Furthermore, more recent studies have shown that applying ROS scavengers, such as polyphenols, to osteoclast precursors attenuate osteoclastogenesis [31–33]. The loss of Casp-2 has been shown to promote increased levels of total cellular ROS as well as mitochondrial superoxide in certain cell types [21,34]. During osteoclast differentiation, we showed that the loss of Casp-2 resulted in increased ROS levels after RANKL addition, but was not evident in BMMs. Although the catalase level in BMMs was significantly reduced and SOD2 appeared lower in the *Casp2*$^{-/-}$ cells, this appears consistent with the minor, non-significant increases in ROS in BMMs seen in Fig. 7. SOD2 is the main antioxidant in the mitochondria that reduces superoxide into H_2O_2. The superoxide production can be detected by the MitoSOX assay. In macrophages, SOD2 is lower in *Casp2*$^{-/-}$ cells, but not significantly different, which agrees with the higher but not significantly different results from the MitoSOX assay. This data indicates that superoxide levels are increased in *Casp2*$^{-/-}$ cells (although not significantly at D3) while SOD2 levels are reduced throughout the differentiation process. Superoxide may be transported out of the mitochondria [35] for reduction by other antioxidants, such as SOD1, or possibly directly affect signaling pathways in the cytosol to increase osteoclast differentiation. However, it is currently uncertain in the literature regarding whether superoxide, H_2O_2, or both function as the critical ROS factors to induce osteoclast differentiation. Catalase, on the other hand, is present in the cytosol and is one antioxidant that can reduce H_2O_2. In M0 cells, catalase levels were significantly decreased in *Casp2*$^{-/-}$ cells compared to WT while the total ROS levels per the DCFDA assay were not significantly different. A possible reason for this is compensation by other antioxidants with similar function to catalase within the cytosol. In addition, as the DCFDA assay detects total ROS within the cell, the lower levels within the unstimulated macrophages may be attributed to other forms that would not be reduced by catalase. Following RANKL administration other studies have shown that there is an increase in ROS including H_2O_2, which would then be available to be reduced by catalase. The overall increase at D1 can be attributed to the increase in ROS following RANKL administration. As the early differentiation markers seen in Fig. 6 have been shown to be increased over controls in condition containing higher ROS such as H_2O_2 [5,6], the lack of a significant difference suggests that initial ROS production following RANKL is not significantly different between *Casp2*$^{-/-}$ and WT cells. Therefore, the increase seen at D1 is most likely due to the decreased reduction of ROS by the antioxidant enzymes including SOD2 and catalase as they are significantly lower in pre-osteoclasts treated with RANKL. At D2, however, we do not see any significant difference in total ROS levels, perhaps due to compensation by other antioxidants at that time point. Then the increase that is seen at D3 may possibly be attributed to the additive increase in superoxide production that is occurring at that time that is higher in *Casp2*$^{-/-}$ cells compared to WT although not significantly so. The increased contribution of mitochondrial ROS makes sense at this point as the later

stages of differentiation are associated with increased mitochondrial biogenesis that serves to increase overall ROS levels and assist in NFATc1 autoamplification. The data presented here indicate that the increased total and especially mitochondrial ROS in *Casp2*$^{-/-}$ cells helps promote osteoclast differentiation. With regard to the origin of the increased ROS, in agreement with data presented by Shalini et al. [21], it appears to be driven by a decreased capacity to scavenge ROS as shown from a reduction in FoxO3a and the antioxidants it controls including catalase and SOD2. An alternate pathway that may be implicated in Casp-2 actions during osteoclastogenesis pertains to caspase-3 (Casp-3) as this caspase has also been implicated as a mediator of osteoclastogenesis [36,37]. Casp-2 has been shown to activate Casp-3 upstream of mitochondrial cytochrome c release during cellular apoptosis, but evidence suggests that Casp-2 may also be cleaved and activated by Casp-3 [17,38]. However, as the mutual activation of Casp-2 and -3 involves the cleavage of Casp-2, which was not detected in this study, this pathway does not appear to be involved. Together, the data from these studies suggest that that ROS especially from mitochondria is crucial during the late stages of osteoclastogenesis, possibly promoting NFATc1 auto-amplification and osteoclast gene transcription. The involvement of mitochondria during osteoclastogenesis is an important component, which requires even further study.

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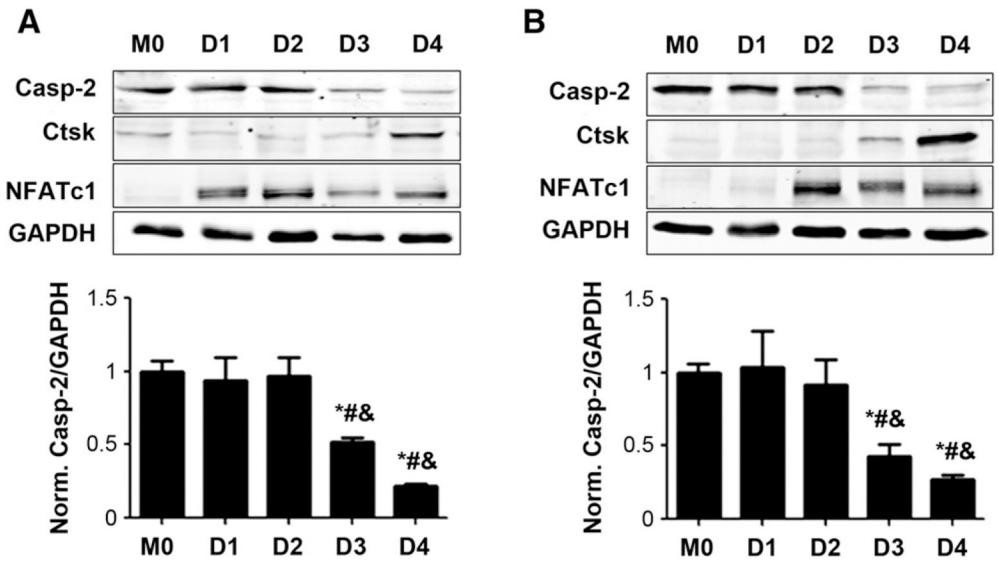

Fig. 1.

Casp-2 protein levels decline during osteoclast differentiation. RAW 264.7 (A) and primary BMMs (B) were cultured with RANKL to induce osteoclastogenesis and cell lysates were prepared at each day of differentiation. Western blots were probed with antibodies against Casp-2, NFATc1, CtsK and GAPDH. Casp-2 levels were quantified and normalized to GAPDH and then to day 0 results, or BMM (M0), control to highlight the decline in Casp-2 protein from baseline. Significant differences in the sample compared to other days were denoted as follows: M0 (*), D1 ($^{\#}$), and D2 ($^{\&}$). ($P < 0.05$; n = 3).

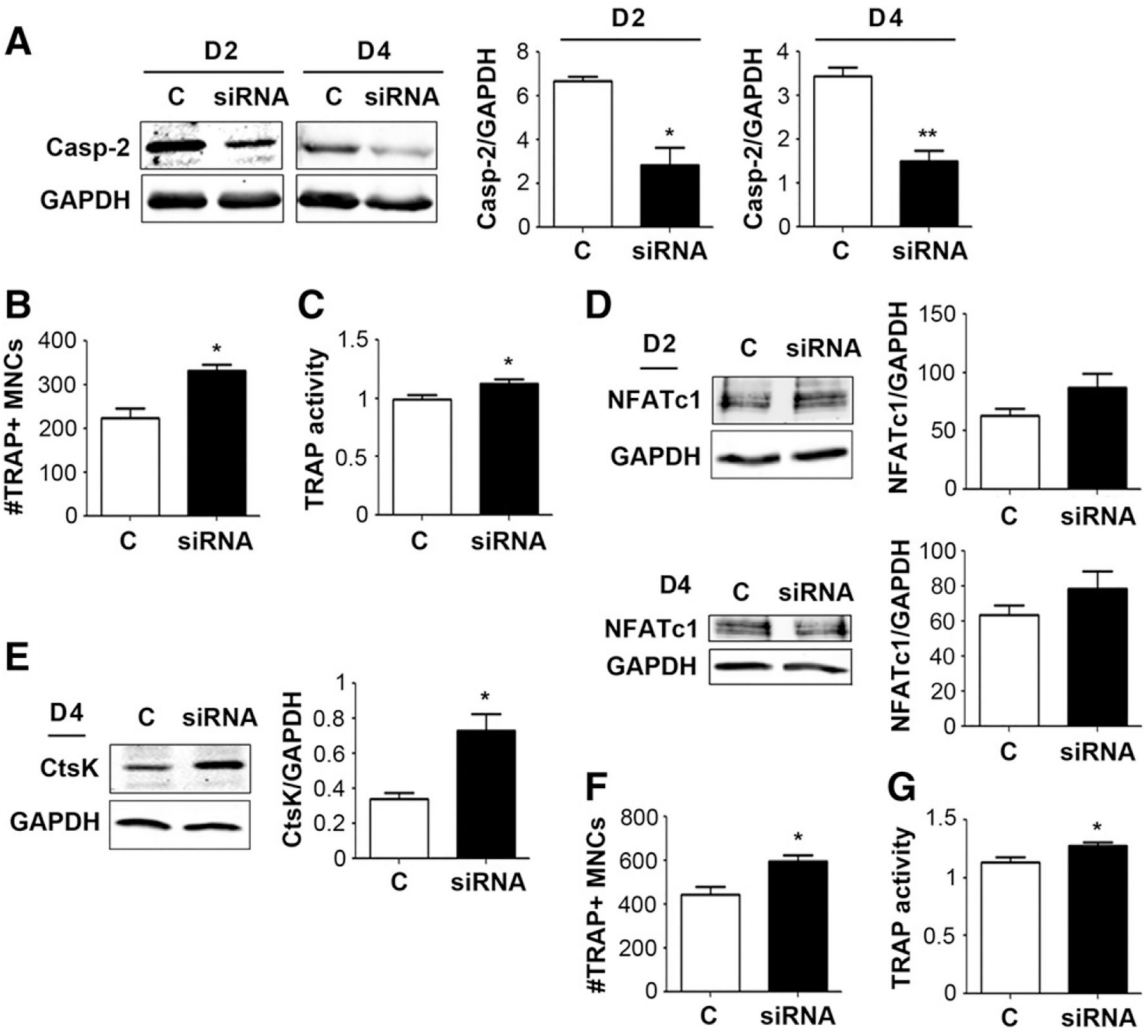

Fig. 2.

Knockdown of Casp-2 promotes osteoclast differentiation. RAW 264.7 cells were treated with Casp-2 siRNA or scrambled control siRNA as well as with RANKL to induce osteoclastogenesis. (A) Cell lysates from both pre-osteoclast (D2) and osteoclast (D4) samples and were immunoblotted with anti-Casp-2 or GAPDH antibody. (B) Osteoclast numbers were evaluated by counting the total number of $TRAP^+$ multinucleated cells (MNCs) in each well of a 48 well plate. (C) TRAP activity in samples containing siRNA compared to control was also quantified. (D) Cell lysates from both pre-osteoclast (D2) and osteoclast (D4) samples were immunoblotted with anti-NFATc1 or GAPDH. (E) Cell lysates from osteoclasts were immunoblotted with anti-CtsK or GAPDH. siRNA against Casp-2 was added only at day 2 and both numbers of $TRAP^+$ MNCs (F) and TRAP activity (G) were analyzed. (n = 3–6; *, $P < 0.05$; **, $P < 0.01$).

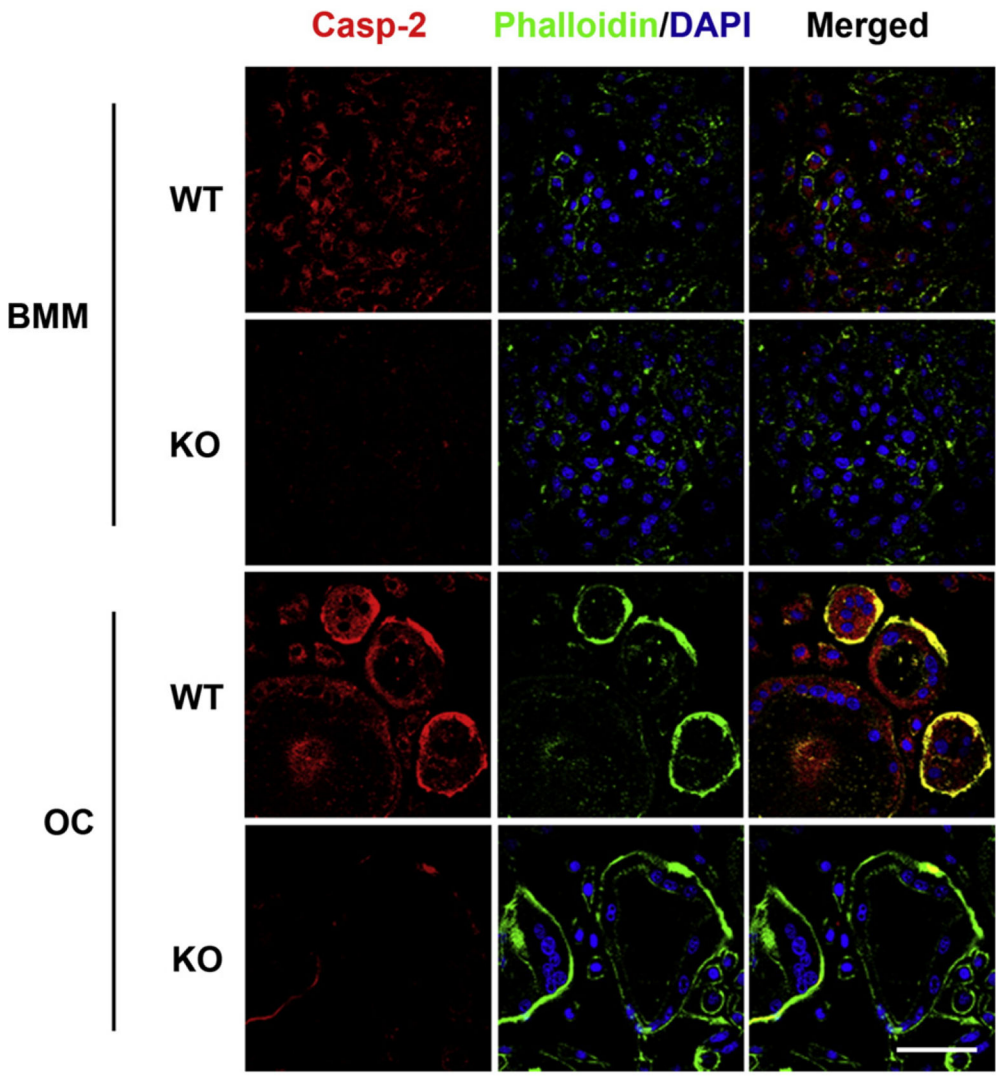

Fig. 3.

BMM and osteoclasts from *Casp2*$^{-/-}$ mice do not express Casp-2. BMMs were isolated from both WT and *Casp2*$^{-/-}$ animals and some cells were induced to differentiate into osteoclasts via RANKL addition. Cells were labeled with anti-Casp-2 antibody (left panels; red) and counter stained with phalloidin (green) to detect F-actin and DAPI (blue) for nuclei visualization (center panels). Merged images are shown in right panels. Bar, 30 μm.

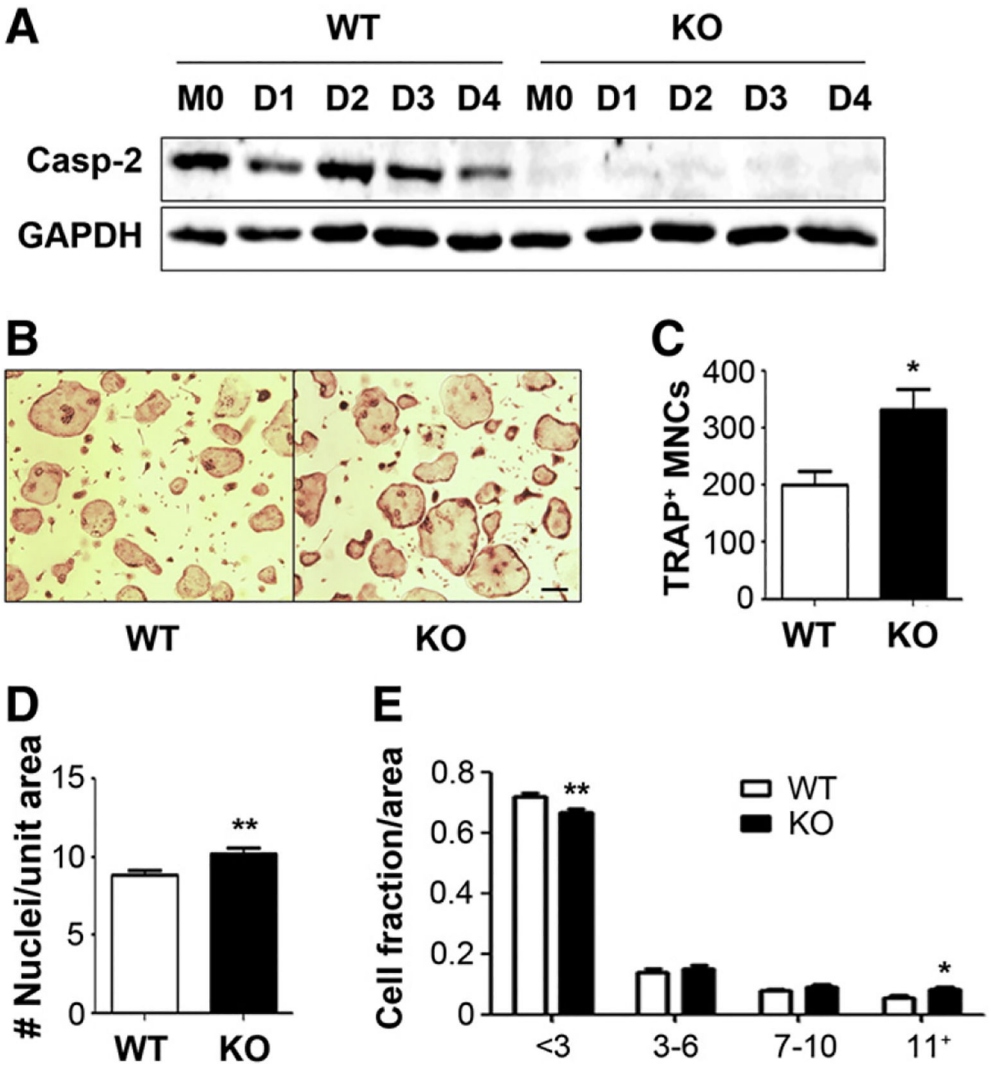

Fig. 4.

The loss of Casp-2 results in larger, more numerous osteoclasts. BMMs isolated from WT and *Casp2*$^{-/-}$ (KO) animals were induced to undergo osteoclastogenesis via RANKL addition. (A) Cell lysates were collected at each day of the differentiation process and immunoblotted with anti-Casp-2 or GAPDH antibody. (B) Representative images were taken of the osteoclast cultures derived from WT and *Casp2*$^{-/-}$ (KO) animals. (C) Multinucleated (MNC) TRAP-positive osteoclast numbers from *Casp2*$^{-/-}$ cells were quantified and, (D) the ratio of #nuclei/unit (cell) area was calculated. (E) Cells in culture were categorized according to the number of nuclei per cell compared between WT and *Casp2*$^{-/-}$ (KO) cells. Bar, 100 μm (n = 3; *, $P < 0.05$; **, $P < 0.01$).

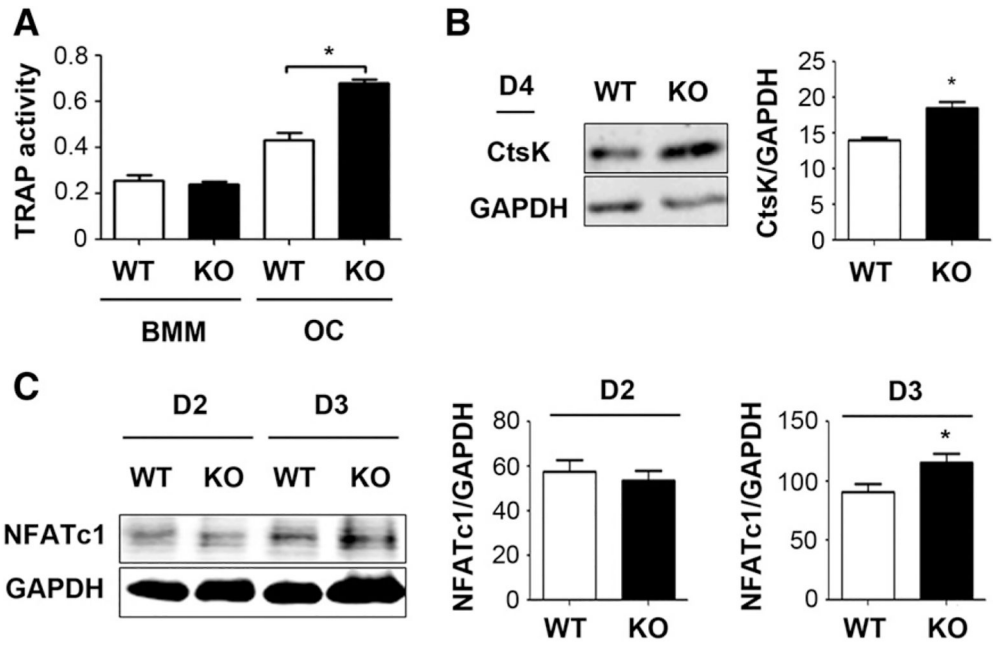

Fig. 5.

Increased TRAP activity and osteoclast markers associated with the loss of Casp-2. (A) Both BMM and osteoclasts (OC) from WT and *Casp2*$^{-/-}$ (KO) animals were assessed for TRAP activity. (B) Cell lysates from WT and *Casp2*$^{-/-}$ (KO) osteoclast cultures were immunoblotted with anti-CtsK or GAPDH antibody and the ratio of CtsK to GAPDH was quantified (right panel). (C) Cell lysates at both day 2 and day 3 of culture were immunoblotted with anti-NFATc1 or GAPDH antibody and the ratio of NFATc1 to GAPDH at day 2 and day 3 was quantified (right panels). (n = 3; *, $P < 0.05$).

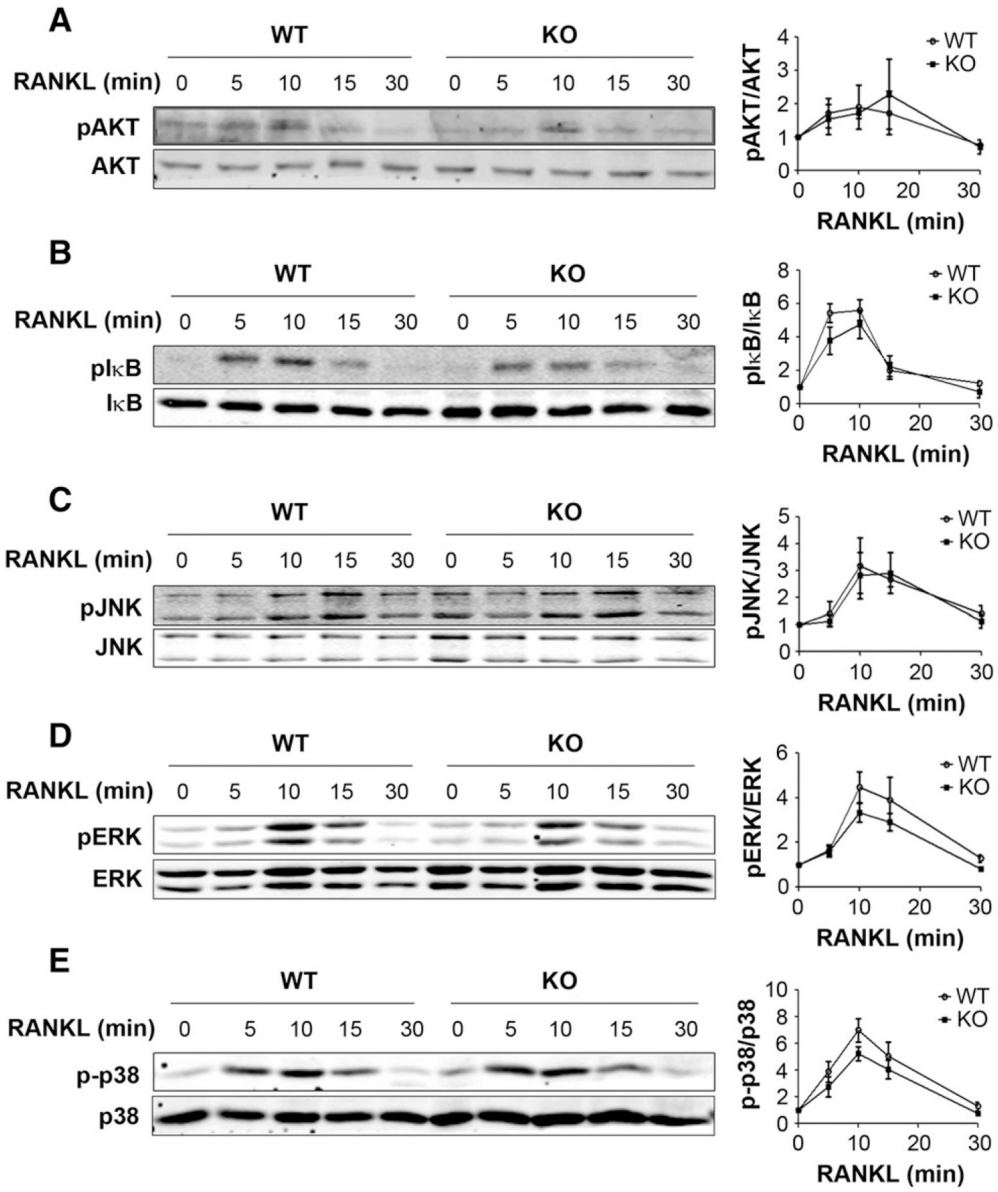

Fig. 6.

The loss of Casp-2 does not affect markers of early differentiation. BMMs collected from WT and *Casp2*$^{-/-}$ animals were serum-starved for 6 h, and then loaded with 100 ng/mL RANKL. Cell lysates were collected at 0, 5, 10, 15, and 30 min and were immunoblotted with anti- phosphorylated and total (A) Akt, (B) IκBα, (C) JNK, (D) ERK, and (E) p38 or corresponding GAPDH antibody. The ratio of phosphorylated to total forms of the evaluated proteins was quantified and normalized to non-RANKL treated control. (n = 3–4).

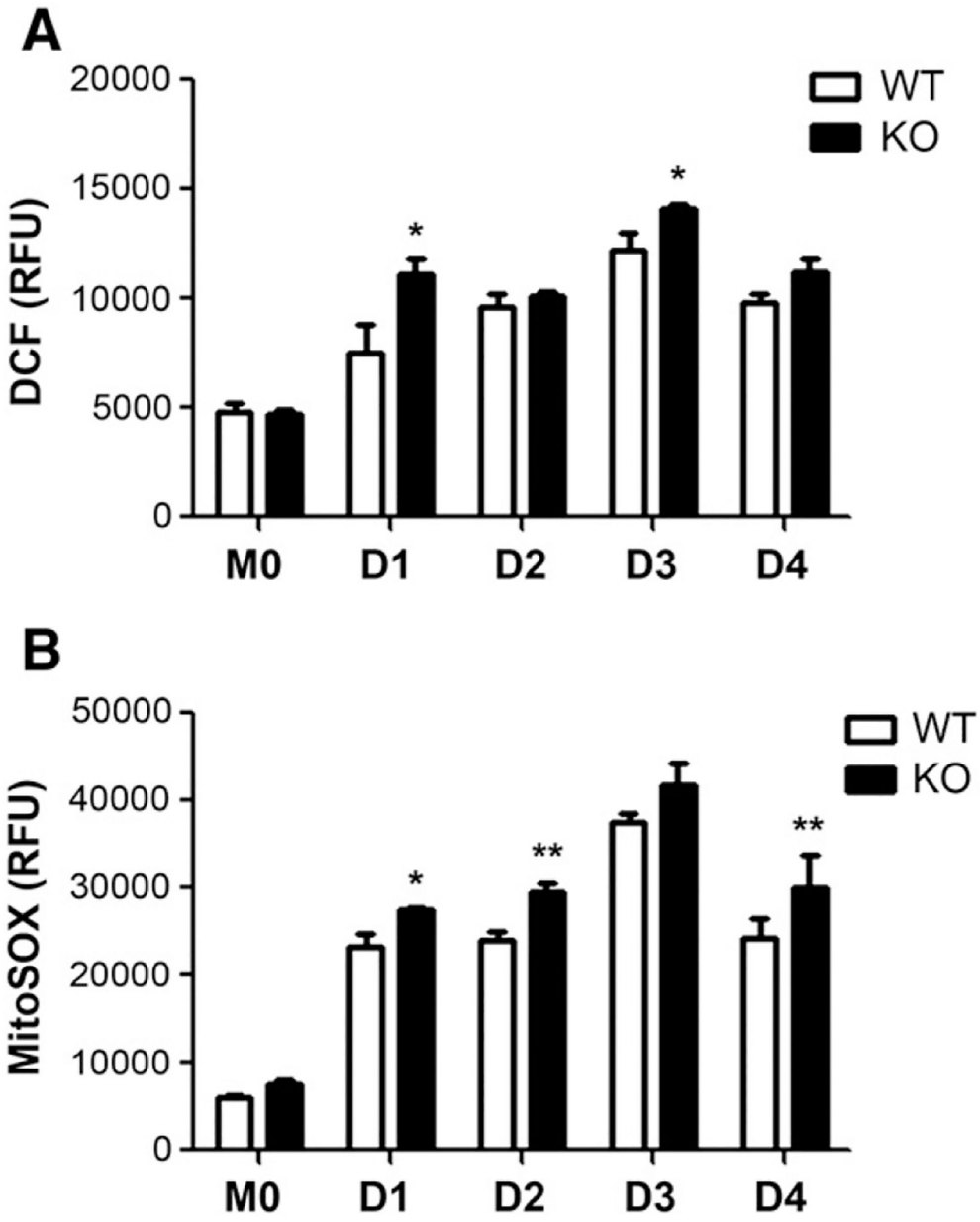

Fig. 7.

Higher total ROS and mitochondrial superoxide production after RANKL administration in cells lacking Casp-2. (A) Total cellular ROS was assessed by DCF fluorescence and (B) mitochondrial superoxide production in WT and *Casp2*$^{-/-}$ (KO) cells during each day of osteoclast differentiation. (n = 6; *, $P < 0.05$; **, $P < 0.01$).

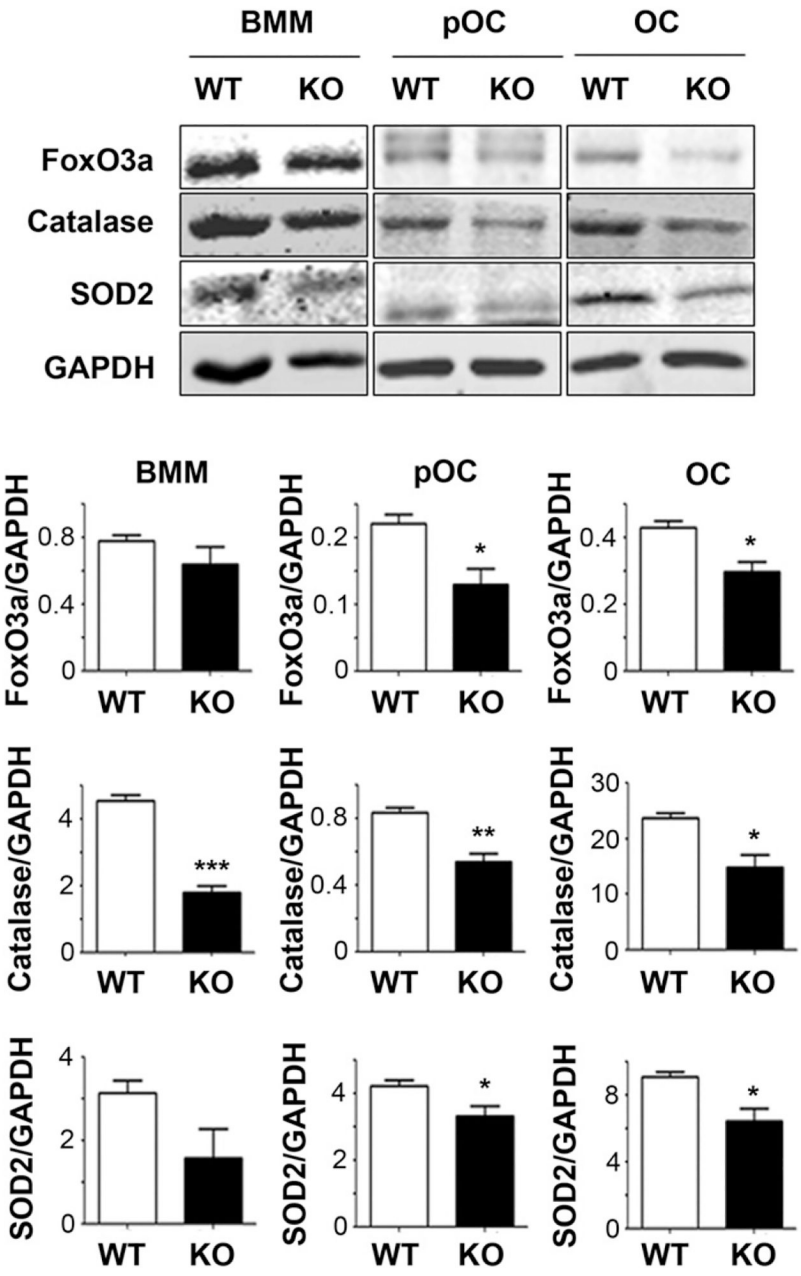

Fig. 8.

Pre-osteoclasts and osteoclasts have lower levels of antioxidants regulated by FoxO3a in *Casp2*$^{-/-}$ mice. Cell lysates collected from BMMs, pre-osteoclasts (pOCs) and osteoclasts (OCs) from WT and *Casp2*$^{-/-}$ (KO) animals were immunoblotted with antibodies against FoxO3a and its target antioxidants, catalase and SOD2. The ratio of FoxO3a, catalase or SOD2 to GAPDH was quantified by densitometry (lower panels). (n = 3; *, $P < 0.05$; **, $P < 0.01$).