Published in final edited form as:

Ann N Y Acad Sci. 2016 January ; 1364(1): 52–61. doi:10.1111/nyas.12709.

Enhanced osteoclastogenesis by mitochondrial retrograde signaling through transcriptional activation of the cathepsin K gene

Manti Guha^{1,a}, Satish Srinivasan^{1,a}, Alexander Koenigstein¹, Mone Zaidi², and Narayan G. Avadhani¹

¹Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

²The Mount Sinai Bone Program, Mount Sinai School of Medicine, New York, New York

Abstract

Mitochondrial dysfunction has emerged as an important factor in wide ranging human pathologies. We have previously defined a retrograde signaling pathway that originates from dysfunctional mitochondria (Mt-RS) and causes a global nuclear transcriptional reprogramming as its endpoint. Mitochondrial dysfunction causing disruption of mitochondrial membrane potential and consequent increase in cytosolic calcium $[Ca^{2+}]_c$ activates calcineurin and the transcription factors NF- κ B, NFAT, CREB, and C/EBP δ . In macrophages this signaling complements receptor activator of nuclear factor kappa-B ligand (RANKL)–induced osteoclastic differentiation. Here, we show that the Mt-RS activated transcriptional coactivator heterogeneous ribonucleoprotein A2 (hnRNP A2) is induced by hypoxia in murine macrophages. We demonstrate that the cathepsin K gene (*Cstk*), one of the key genes upregulated during osteoclast differentiation, is transcriptionally activated by Mt-RS factors. HnRNP A2 acts as a coactivator with nuclear transcription factors, cRel, and C/EBP δ for *Cstk* promoter activation under hypoxic conditions. Notably, our study shows that hypoxia-induced activation of the stress target factors mediates effects similar to that of RANKL with regard to *Cstk* activation. We therefore suggest that mitochondrial dysfunction and activation of Mt-RS, induced by various pathophysiologic conditions, is a potential risk factor for osteoclastogenesis and bone loss.

Keywords

hypoxia; mitochondrial retrograde signaling; *Cstk* promoter; hnRNP A2; osteoclastogenesis

Address for Correspondence: Narayan G. Avadhani, 3800 Spruce Street, Rm # 189E, Department of Animal Biology, School of Veterinary Medicine, University of Philadelphia, PA 19104. narayan@vet.upenn.edu.

^aThese authors contributed equally to this work.

Author contributions

MG, SS and NGA designed the study; MG, SS, AK performed experiments; MG, SS and NGA analyzed data and wrote the manuscript; MZ provided reagents and critical suggestions in the design of this study.

Conflicts of interest

The authors have no conflict of interest in the work.

Introduction

Bone mass is tightly regulated by a delicate balance between osteoblastic bone formation and osteoclastic bone resorption. An imbalance of this process leads to osteoporosis and bone loss.¹ Osteoporosis is a major health risk worldwide and is a critical determinant of the fracture risk assessment. Osteoclasts are giant bone resorbing cells derived from monocyte-macrophage lineage by multistep processes that involve activation of multiple signaling pathways.² Osteoclastogenesis itself is a highly regulated process and is controlled by both metabolic and hormonal factors.^{3,4} Several recent studies show that cellular hypoxia is an important pathophysiological condition that increases osteoclast differentiation of macrophages.^{5–7} Low oxygen tension leading to hypoxia is commonly encountered by cells in various pathologies like ischemia, cancer, rheumatoid arthritis and fracture. All of these conditions are associated with bone loss.^{8–11} Mitochondria are one of the direct cellular targets of hypoxia. We and others have previously reported that mitochondrial dysfunction under hypoxia is associated with elevated mitochondrial reactive oxygen species (ROS).¹² We have shown that hypoxia induces a Ca^{2+} /calcineurin-dependent retrograde signaling pathway that augments osteoclastogenesis in murine macrophages.⁶ Moreover, the signature transcription factors of the Ca^{2+} /calcineurin-dependent mitochondrial retrograde signaling pathway (Mt-RS), NF- κ B, NFAT, CREB, and C/EBP δ , were elevated during hypoxia-induced osteoclastogenesis.⁷

Hypoxic conditions induce the expression of key markers of osteoclast formation, markers such as cathepsin K, TRAP, and the calcitonin receptor.⁷ Cathepsin K is a highly abundant cysteine protease expressed in osteoclasts and reported to be involved in bone matrix degradation that leads to bone resorption;^{13–15} it is selectively expressed in osteoclasts, and under acidic conditions degrades type I collagen. Complete deficiency of cathepsin K activity leads to pycnodysostosis, a severe and rare autosomal recessive bone sclerotic disorder, and cathepsin K-deficient (*Ctsk*^{−/−}) mice are osteopetrotic.¹⁶ In humans, inhibition of cathepsin K is associated with sustained reductions in bone resorption markers, but with smaller and transient reductions in bone formation markers.¹⁷ For these reasons, this protease is an attractive therapeutic target for inhibition of bone resorption.^{17–19}

In the current study we aimed to elucidate the molecular mechanism of activation of the cathepsin K gene (*Ctsk*) by hypoxia-induced Mt-RS, as well as the consequence on osteoclast differentiation from macrophages. We show that hypoxia-induced Mt-RS induces transcription of *Ctsk*, a key marker gene of osteoclasts.²⁰ Activation of the *Ctsk* promoter involves a functional synergy between transcription factors cRel, C/EBP δ , NFAT, and CREB and a novel stress activated coactivator, heterogeneous ribonucleoprotein A2 (hnRNP A2), a protein that shuttles between the cytosol and the nucleus and is involved in mRNA processing and transport.²¹ Previously, we have shown in a number of cell lines that hnRNPA2 is critical for the propagation of the Mt-RS pathway.^{22–24} Our findings below establish a molecular link between mitochondrial dysfunction and osteoclast formation under hypoxic conditions.

Materials and methods

Cell culture

RAW264.7 mouse monocyte macrophages (ATCC, Manassas, VA) were cultured in alpha-modified Eagle's medium supplemented with 10% fetal calf serum, as described previously.⁶ Cells at 70–80% confluence were grown under either hypoxic conditions (5% O₂) or normoxic conditions (21% O₂) for 5 days. Osteoclastogenesis was induced by treating cells with RANKL (25 ng/mL). To study the effect of inhibitors of retrograde signaling on osteoclast formation, cells were treated with FK506 (100 nM), PPP (picropodophyllin, 1 mM), AG1024 (2.5 μM), or genistein (50 μg/mL) along with RANKL (25 ng/mL).

Tartrate resistant acid phosphatase assay

Tartrate resistant acid phosphatase (TRAP) staining was performed using the SIGMA kit (Sigma, St. Louis, MO), according to the manufacturer's instructions. RAW264.7 cells at 30–40% confluence were maintained either at normoxic or hypoxic (5% O₂) condition for 5 days. Fresh medium equilibrated at 5% O₂ was replaced every 2 days. At the end of hypoxia, the adherent cells were fixed with 50% (v/v) ethanol/PBS for 10 min, fixed again with ethanol/acetone (50:50, v/v) for 1 min, and incubated for 10 min at room temperature with the TRAP staining solution. The multinucleated cells (i.e., more than 3 nuclei), which stained dark red, were counted as TRAP-positive osteoclasts.

Cloning of the cathepsin K gene promoter

The murine *Ctsk* promoter DNA (sequence –1000 to +1) (Gene ID: 13038) was amplified from mouse genomic DNA and cloned into the pGL3 mammalian expression vector (Promega, Madison WI) at *Bgl* II and *Hind* III sites. The putative transcription factor binding sites on the murine *Ctsk* promoter were analyzed using MatInspector (www.genomatix.de).

Transfections, gene silencing, and luciferase activity

cRel, C/EBPδ, NFAT, and CREB cDNAs were cloned into pCMV4, and hnRNP A2 cDNA into pCI, as described before.²² Transfections of cDNAs were carried out in cells grown on 6-well dishes using FuGENE® 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN). One μg of promoter DNA construct (pGL3-Cath K) and 0.5 μg of a *Renilla* luciferase construct (Promega), as an internal control, were used in each transfection. Luciferase activity was assayed using the Dual-Luciferase® reporter assay system (Promega). Cotransfections with various cDNAs were carried out using 0.2 μg of each cDNA construct.

For mRNA silencing by transient transfections, predesigned siRNA for mouse C/EBPδ (sc-37723) (Santa Cruz Biotechnology, Santa Cruz, CA), shRNA specific for hnRNPA2, and double-stranded scrambled negative siRNA control (Integrated DNA Technologies, San Diego, CA) were used as described earlier.²² Cells (1 × 10⁶) were transfected with preannealed double-stranded siRNAs for 48 h at a final concentration of 25 nM by reverse transfection as described before.²² Transient transfections were carried out in triplicate by

using siPORT™ NeoFX™ reagent (Life Technologies). After 48 hours, cells were lysed and cathepsin K mRNA expression was assessed by real time PCR.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described before.²² Briefly, 1×10^6 cells were fixed by adding 1% formaldehyde to the culture medium and incubated at 37 °C for 10 min. The cell pellet was suspended in sodium dodecyl sulfate (SDS) lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris (hydroxymethyl) aminomethane, pH 8.1], and the cell lysates were sonicated using an ultrasonic processor sonicator (10 s pulses, 15 times, on ice). Diluted aliquots were precleared using salmon sperm DNA–coupled agarose prior to immunoprecipitations with antibodies (as indicated in the figure) overnight at 4 °C. Preimmune immunoglobulin G was used as a negative control and 10% precleared lysate of each cell type was used as input. Antibody–chromatin complexes were immunoprecipitated by protein A agarose/salmon sperm DNA (50% slurry) (Sigma-Aldrich). The DNA recovered after the reversal of cross-linking was quantified by real-time PCR. Data were analyzed by normalizing with the corresponding input values and expressed as fold-changes with respect to the normoxic cells.

Real time PCR for mRNA quantitation

Total RNA was isolated using TRIzol® reagent according to the supplier's protocol (Life Technologies). The cDNA was generated from 5 µg of RNA using the cDNA High-Capacity Archive kit (Life Technologies, Foster City, CA), and 100 ng of the cDNA was used as template per reaction. SYBR® Green (ABI, Life Technologies) primers were designed for *Cstk* by using the Primer Express® 2.0 software (ABI, Life Technologies).⁶ Data were normalized by comparing β-actin levels as an endogenous control. Results represent three independent experiments.

Statistical analysis

Averages ± standard error (S.E.) of three independent estimates (each taken in triplicate) are presented. Differences between paired variables were determined using two-way analysis of variance (ANOVA). *P* values < 0.05 were considered statistically significant, and *P* values < 0.001 were highly significant.

Results

Hypoxia accentuates the effects of RANKL induced osteoclast formation

Murine macrophages were grown in either normoxic (Fig. 1A, top panel) or under hypoxic condition (Fig. 1A, bottom panel) with and without RANKL to test the induction of osteoclast differentiation. Cells grown under normoxic conditions did not form any osteoclasts. Hypoxic environment by itself was a weak inducer of osteoclasts (Fig. 1A, bottom left panel), whereas hypoxia combined with RANKL treatment induced a higher number of osteoclast-forming cells, as evidenced by TRAP-positive staining (Fig. 1A, bottom right panel).

In a previous study, we showed that Mt-RS activates the PI-3 kinase/IGF1 receptor pathway.^{23, 25} To ascertain the role of Mt-RS in osteoclast formation, macrophages were treated with inhibitors of calcineurin (FK506) or tyrosine kinase (genistein), or with specific IGF1R inhibitors PPP or tryphostin (AG1024). As shown in Figure 1B, treatment with FK506, AG1024, or genistein markedly reduced the number of osteoclasts by about 65%, while treatment with a potent IGF1R-specific inhibitor, PPP, had maximal inhibitory effect (~85%) on differentiation of macrophages to osteoclasts (Fig 1B). These results show that RANKL-induced osteoclastogenesis is mediated by some of the same factors that mediate mitochondrial dysfunction-induced retrograde signaling.

Cstk is one of the key genes activated during osteoclastogenic differentiation from precursor macrophages.^{13–15} We reported earlier that hypoxia induces the expression of *Cstk*.⁶ In agreement with that study, cells subjected to hypoxia showed a 5-fold induction in cathepsin K mRNA expression compared to cells grown under normoxic conditions (Fig. 1C, left panel). Next, we treated the macrophages with RANKL, which potentiates osteoclastic differentiation, and tested the effect of the Mt-RS pathway inhibitors genistein, AG1024, and PPP in modulating cathepsin K mRNA expression. We observed a marked decrease in RANKL-induced *Cstk* expression in hypoxic cells treated with either genistein or PPP, and by about 30% in cells treated with AG1024 (Fig. 1C, right panel).

Hypoxia-induced retrograde signaling factors transcriptionally activate the *Cstk* promoter

We reported earlier in C2C12 skeletal myocytes that hnRNP A2 is a mitochondrial stress-induced transcriptional coactivator and is crucial for the propagation of stress response.²² The immunoblot in Figure 2A shows ~5-fold higher expression of hnRNP A2 protein in the nuclear fraction of RAW264.7 macrophages grown under hypoxic conditions.

One characteristic of Mt-RS responsive promoter is the presence of the four signature factor binding sites immediately upstream of the transcription start site.²² To understand the mechanism of activation of *Cstk* in response to mitochondrial stress, we analyzed the minimal *Cstk* promoter region for consensus sites for binding to the aforementioned transcription factors using the MatInspector algorithm. The cartoon in Figure 2B shows the putative binding sites of the mitochondrial stress target factors on the *Cstk* promoter, which are reminiscent of other Mt-RS response genes we investigated before.^{22,23} We cloned the putative stress responsive *Cstk* promoter in the luciferase reporter vector (pGL3 basic) and assessed promoter activity in macrophages subjected to hypoxia with and without RANKL treatment. Cells grown in parallel under normoxic conditions were used as a control. We observed that the mouse *Cstk* promoter exhibited 2.0-fold higher activity in RAW264.7 macrophage cells grown under hypoxic conditions, compared with cells grown under normoxic conditions (Fig. 2B). In cells subjected to hypoxia, RANKL treatment had only marginal additive effect on *Cstk* promoter activation compared to cells subjected to normoxia. The specificity of the luciferase promoter response to hypoxic stress was confirmed by the lack of transcription activation of pGL3 basic vector under hypoxic conditions (data not shown).

We investigated the contribution of Mt-RS factors in the transcriptional activation of the *Cstk* (sequence –1000 to +1) promoter in RAW264.7 cells by cotransfections with cDNAs

for cRel, C/EBP δ , NFAT, CREB, and hnRNP A2 (Fig. 2B). Relative luciferase activity (normalized to Renilla luciferase as internal control) driven by the *Cstk* promoter was markedly higher in hypoxia-treated cells cotransfected with the above factors. Cotransfections with either NFAT or CREB cDNAs had >2.5 fold higher *Cstk* promoter activation under hypoxia compared to control cells. As expected, hnRNPA2 cDNA alone did not have such effect on *Cstk* promoter activation. However, cotransfections of hnRNPA2 cDNA with cRel and C/EBP δ cDNAs synergistically enhanced luciferase activity (~ 2.3–2.5 fold over either cRel or C/EBP δ cDNAs alone) in cells subjected to hypoxia, suggesting cooperativity between these factors. In cells grown under normoxic conditions, however, we observed only a minimal increase in *Cstk* promoter activity by cotransfections with these cDNAs.

As mentioned earlier, RANKL is considered to be an obligate factor for osteoclast formation; and hypoxia by itself is insufficient for robust induction of osteoclasts. However, in our cotransfection experiments carried out with added RANKL, we observed that RANKL had only a marginal effect on *Cstk* promoter activation when added to cells under hypoxic conditions (Fig. 2C).

In vivo association of Mt-RS factors with the *Cstk* promoter

To ascertain the *in vivo* association of stress-activated transcription factors and hnRNP A2 at the target promoters, we performed ChIP assays in Raw264.7 cells grown under normoxic and hypoxic conditions. ChIP analysis in Figure 3A shows increased association of hnRNP A2, cRel, C/EBP δ , and NFAT with the *Cstk* promoter in cells grown under hypoxic conditions. The increased occupancy of Mt-RS factors to the *Cstk* promoter under hypoxic conditions is in agreement with our cotransfection studies demonstrating the involvement of these factors in promoter activation.

We further assessed the contribution of the retrograde signaling activated transcription factors C/EBP δ and hnRNP A2 in the transcription activation of Cathepsin K promoter using a siRNA approach (Fig. 4B). Cathepsin K mRNA was reduced by >40% by siRNA targeted silencing of either C/EBP δ or hnRNPA2 (Fig. 3B). These results suggest that NF- κ B, C/EBP δ , CREB, and NFAT, along with hnRNPA2 are important factors in mediating transcriptional activation of *Cstk* under hypoxia-induced Mt-RS.

Discussion

Mitochondria are considered the power house of the cell since they generate most of cellular ATP. In addition they have important roles in calcium homeostasis, integration of metabolism and regulation of apoptosis. It is therefore predictable that mitochondrial dysfunction could have severe consequences to cell growth and survival. Previous studies from our laboratory have shown that mitochondrial dysfunction caused by loss of mtDNA or loss of membrane potential initiates a calcium/calcineurin-dependent retrograde signaling pathway that transcriptionally activates several stress target genes and transforms the cells to invasive, tumorigenic phenotype.^{26–28} Interestingly, the serine/threonine phosphatase calcineurin, activated by multiple pathways including the Mt-RS, is an established inducer of osteoclastic differentiation from macrophages.^{29,30} In an analogous study, we showed

that hypoxia-mediated mitochondrial dysfunction induces differentiation of murine macrophage RAW264.7 cells to osteoclasts, likely by activating the same Mt-RS.^{6,7} When treated with the calcineurin inhibitor FK506, cells grown under hypoxic conditions markedly inhibited osteoclast formation (Fig. 1B), suggesting that common upstream propagators such as calcineurin initiate Mt-RS and osteoclastic differentiation.

In our earlier studies, we showed that in RAW264.7 cells activation of Mt-RS depends on mitochondrial ROS. In fact, earlier work showed that differentiation of macrophages to osteoclasts brought about by RANKL is mediated by ROS.^{31–34} Furthermore, hypoxia-induced differentiation of cultured macrophages to osteoclast-like multinucleated cells was markedly inhibited by mitochondria targeted with the antioxidant Mito-Q, suggesting a role for mitochondrial ROS in osteoclastogenesis.⁷ A recent report also showed that accumulation of mitochondrial hydrogen peroxide was essential for osteoclast differentiation, and that mitochondria-targeted catalase was effective in preventing bone resorption caused by loss of estrogen.³⁵

The mechanism by which hypoxia and mitochondrial stress activates osteoclastic differentiation remains unclear. A recent study demonstrated that stabilization of the hypoxia inducible factor (HIF)-1 α under hypoxia leads to activation of osteoclasts.³⁶ However, another report showed that mitochondrial complex I subunit *Ndufs4* is required for osteoclastogenesis, and deletion of *Ndufs4*, and the resultant mitochondrial dysfunction, abrogate osteoclast formation.³⁷

Our previous work has shown that hypoxia-induced mitochondrial dysfunction activates stress-induced transcription factors such as CREB, C/EBP δ , p50, and NFAT2.^{6,7} However, the contribution of these transcription factors in hypoxia-mediated osteoclast differentiation has remained unclear. Cathepsin K, primarily responsible for the degradation of bone matrix by osteoclasts, remains a highly pursued molecular target, e.g., inhibitors of cathepsin K are being developed for treatment of osteoporosis.^{17–19}

In the study here, we show that cRel, C/EBP δ , NFAT, and hnRNP A2 (the latter being an established mitochondrial stress activated transcriptional co-activator²²) have roles in activation of the *Cstk* promoter. Transcriptional control of osteoblast differentiation involving NF- κ B, NFAT, Runx, Msx2, Osx, and Foxo factors from mesenchymal progenitor cells has been described.¹ The data presented here extends previous reports and shows the involvement of additional factors—CREB, C/EBP δ , NFAT, and, particularly, hnRNP A2—in modulating osteoclastogenesis by transcriptionally regulating *Cstk* expression.

Numerous factors have been implicated to play a causal role in osteoporosis, and low estrogen is a major contributor. In recent years, other factors such as dietary calcium, parathyroid function, and genetic background have been shown to be critical determinants, although their contribution remains unclear. Our studies suggest that the mechanism of RANKL signaling involved in osteoclastogenesis mimics the mitochondrial retrograde signaling initiated by hypoxic stress (Fig. 4). Therefore, designing specific inhibitors to abrogate Mt-RS might prove beneficial in halting bone loss, providing a therapeutic advantage in patients suffering from osteoporosis.

Acknowledgments

This work was supported by NIH Grants CA22762 (to N.G.A.), AR067066 (to M.Z. and N.G.A.) and The Harriet Ellison Woodward Trust fund (to N.G.A.).

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Figure 1.

Hypoxia augments the formation of RANKL-mediated TRAP-positive osteoclasts. (A) Osteoclasts formed from RAW264.7 cells grown under hypoxic conditions (5% O₂) for 5 days with or without addition of RANKL (25 ng/mL). (B) Effect of inhibitors of Mt-RS on RANKL-induced osteoclast formation under hypoxic conditions. The inhibitors FK506 (100 nM), PPP (1 μM), AG1024 (2.5 μM), or genistein (50 μg/mL) were added at the time of addition of RANKL. (C) Cathepsin K mRNA levels assessed by RT-PCR in RAW264.7 cells grown under either normoxic or hypoxic conditions (left panel), and in the presence of

RANKL and Mt-RS inhibitors under hypoxic conditions (right panel). *, $P \leq 0.05$; **, $P \leq 0.001$.

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Figure 2.

Transcriptional activation of the *Ctsk* promoter by hypoxia-induced mitochondrial stress factors. (A) Immunoblot showing the induction of the mitochondrial stress activated coactivator hnRNP A2 in the nuclear fraction of RAW264.7 cells. (B) *Top*: cartoon showing the putative stress activated factor binding sites on the murine *Ctsk* promoter. *Bottom*: relative promoter activity (normalized to *Renilla* luciferase activity) in RAW264.7 cells grown under either normoxic or hypoxic conditions (5% O₂) for 3 days. Cotransfection of *Ctsk* promoter and Mt-RS-activated transcription factors in control and hypoxia-treated

RAW264.7 cells. (C) Cotransfection of *Ctsk* promoter and Mt-RS-activated transcription factors in control and hypoxia-treated RAW264.7 cells treated with RANKL. *, $P = 0.05$; **, $P = 0.001$

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Figure 3.

In vivo functional association of Mt-RS factors to *Ctsk* expression. (A) ChIP analysis of the *Ctsk* promoter with hnRNP A2, cRel, C/EBP δ , and NFAT antibodies in normoxic and hypoxic cells. Preimmune serum was used as a negative control. Real-time PCR analysis was carried out using 10% input DNA for normalization. Values are expressed as fold-changes relative to the factor binding in normoxic cells. (B) RT-PCR showing cathepsin K mRNA levels in RAW264.7 cells expressing siRNA to Mt-RS factors C/EBP δ and hnRNP A2 under hypoxic conditions (5% O₂).

Figure 4. Schematic outline showing the mechanism by which hypoxia induced mitochondrial retrograde signaling in RAW264.7 cells activates *Ctsk* and osteoclastic differentiation.