Dynamin-Related Protein 1 Inhibition Attenuates Cardiovascular Calcification in the Presence of Oxidative Stress

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ABSTRACT

**Rationale:** Mitochondrial changes occur during cell differentiation and cardiovascular disease. Dynamin-related protein 1 (DRP1) is a key regulator of mitochondrial fission. We hypothesized that DRP1 plays a role in cardiovascular calcification, a process involving cell differentiation and a major clinical problem with high unmet needs.

**Objective:** To examine the effects of osteogenic promoting conditions on DRP1, and whether DRP1 inhibition alters the development of cardiovascular calcification.

**Methods and Results:** DRP1 was enriched in calcified regions of human carotid arteries, examined by immunohistochemistry. Osteogenic differentiation of primary human vascular smooth muscle cells (SMCs) increased DRP1 expression. DRP1 inhibition in human SMCs undergoing osteogenic differentiation attenuated matrix mineralization, cytoskeletal rearrangement, mitochondrial dysfunction, and reduced type I collagen secretion and alkaline phosphatase activity. DRP1 protein was observed in calcified human aortic valves, and DRP1 RNA interference reduced primary human valve interstitial cell calcification. Mice heterozygous for Drp1 deletion did not exhibit altered vascular pathology in a PCSK9 gain-of-function atherosclerosis model. However, when mineralization was induced via oxidative stress, DRP1 inhibition attenuated mouse and human SMC calcification. Femur bone density was unchanged in mice heterozygous for Drp1 deletion, and DRP1 inhibition attenuated oxidative stress-mediated dysfunction in human bone osteoblasts.

**Conclusions:** We demonstrate a new function of DRP1 in regulating collagen secretion and cardiovascular calcification, a novel area of exploration for the potential development of new therapies to modify cellular fibrocalcific response in cardiovascular diseases. Our data also support a role of mitochondrial dynamics in regulating oxidative stress-mediated arterial calcium accrual and bone loss.

**Keywords:** Mitochondria, dynamin-related protein 1, DNM1L, calcification, collagen, oxidative stress, aortic valve calcification, atherosclerosis.
Nonstandard Abbreviations and Acronyms:

AAV                adeno associate virus
ApoE−/−             apolipoprotein E-deficient
COL1A1          type 1 collagen
CM             control media
DNM1L         dynamin 1 like
DRP1            dynamin-related protein 1
ER             endoplasmic reticulum
H2O2           hydrogen peroxide
Ldlr−/−     low-density lipoprotein receptor-deficient
M-CSF            macrophage colony-stimulating factor
Mdivi-1      mitochondria division inhibitor-1
Mfn2              mitofusin 2
MSX2              Msh homeobox 2
OM          osteogenic media
PBMCs            peripheral blood mononuclear cells
PCSK9          proprotein convertase subtilisin/kexin type 9
PPi                pyrophosphate
PKC            protein kinase c
RANKL        receptor activator of nuclear factor kappa-B ligand
RUNX2        runt-related transcription factor 2
SMCs             smooth muscle cells
SOX9             SOX gene family member 9
TNAP        tissue non-specific alkaline phosphatase
TMRE       tetramethylrhodamine, ethyl ester
TRAP            tartrate-resistant acid phosphatase
VICs             valve interstitial cells

INTRODUCTION

Mitochondria exist in dynamic networks where they are continuously generated, joined through fusion, and divided by fission, with dynamin-related protein 1 (DRP1) being a key protein allowing mitochondria to multiply or be degraded through mitochondrial fission. Mitochondrial activity, shape, and localization have been suggested to regulate cell differentiation by influencing programs that control cell fate. Differentiation is a characteristic phenotypic feature of vascular smooth muscle cells (SMCs), with phenotypic modulation of vascular cells allowing for the vasculature to efficiently perform and respond to different physiological and pathological conditions. One such condition with high unmet needs involves SMCs and valve interstitial cells (VICs) undergoing differentiation into osteoblast-like cells. The resulting arterial and aortic valve accumulation of calcium mineral deposits promotes major adverse clinical events.

Mitochondrial dynamics are associated with many biological processes related to cardiovascular calcification including: calcium homeostasis, cellular differentiation, apoptosis, oxidative stress, autophagy, and exocytosis. Mice with a Drp1 middle domain C425F dominant-negative mutation develop cardiomyopathy along with spotty calcifications in heart tissue. No mechanism has been suggested for the presence of cardiac calcification in these mice, and whether DRP1 regulates calcification of other tissues, including the vasculature is unknown. DRP1 inhibition prevents rat vascular neointima formation in a
balloon injury model\textsuperscript{12}, suppresses lesion formation in diabetic ApoE-deficient mice\textsuperscript{13}, and oxidized low density lipoprotein induces DRP1 mediated mitochondrial fragmentation in human SMCs\textsuperscript{14}, suggesting a possible role of DRP1 in vascular disease. Reduced mitochondrial fusion protein, Mitofusin 2 (MFN2) that acts in an opposite function to that of DRP1 driven mitochondrial fission, is associated with atherosclerosis pathology in mice\textsuperscript{15,16}, rabbits\textsuperscript{17}, and humans\textsuperscript{16}. Overexpression of \textit{Mfn2} in atherosclerotic rabbits reduces lesion formation\textsuperscript{17}. Taken together these studies suggest a role of mitochondrial dynamics in atherosclerosis, a disease highly associated with calcification. Mutations in the dynamin 1 like gene (DNM1L) encoding the DRP1 protein, including nonsense and dominant negative mutations, have been reported in a small number of human patients\textsuperscript{18-24}. While these patients exhibited a wide range of pathologies, from normal early development to neuronal development defects, cardiovascular calcification has not been reported. As such, an involvement of DRP1 in human soft tissue calcification remains to be demonstrated. Therefore, we sought to examine whether DRP1 plays a role in human cardiovascular calcification, and if its inhibition alters the calcification process.

\textbf{METHODS}

Detailed methodology is included in the Online Supplemental Material.

\textbf{RESULTS}

\textit{DRP1 localized to calcified areas of human carotid arteries.}

To analyze DRP1 expression in human vascular disease conditions, we performed DRP1 immunostaining on human carotid atherosclerotic plaques ($N=5$). DRP1 immunoreactivity was high in tissues with calcification, and more specifically around calcified areas (Figure 1A and Online Figure I). DRP1 was observed in SMCs (HHF-35, \textit{\alpha}SMA) and some macrophages (CD68) in calcified human arteries (Figure 1C and D). SMC mitochondrial area was enlarged in calcified compared to non-calcified regions of carotid artery tissue from patients undergoing endarterectomy ($N=3$), examined by electron microscopy (Figure 1E).

\textit{Increased DRP1 in calcifying human SMCs associated with mitochondrial alterations}

An established cell culture vascular calcification model\textsuperscript{25}, promotes the transition of SMCs to an osteoblast-like cell phenotype. \textit{\alpha}SMA positive (Online Figure IIA) SMCs cultured under osteogenic conditions have increased activity of a key enzyme, tissue non-specific alkaline phosphatase (TNAP), peaking at two weeks in culture, and develop a mineralized matrix at three weeks in culture. Osteogenic transition of primary human coronary artery SMCs increased (approximately two-fold) DRP1 protein and mRNA (Figure 2A). To assess mitochondrial morphology in this model, mitochondria were labeled using MitoTracker Red and live cell mitochondrial imaging analysis was performed with confocal microscopy. Human SMC mitochondria appeared to be more fragmented when cultured in osteogenic media (OM) compared to control media (CM) (Figure 2B). We confirmed these observations through quantifying mitochondrial aspect ratios, a measurement of the major mitochondrial axis to the minor axis.

Mitochondrial aspect ratios were reduced in two-week-OM cultured cells compared to CM conditions (Figure 2B). To test whether this change was due to DRP1 activity or protein content, the selective small molecule DRP1 inhibitor, mitochondrial division inhibitor 1 (Mdivi-1) was added at a concentration (50 $\mu$mol/L) previously reported to fully inhibit DRP1 activity in cells\textsuperscript{26}. Mdivi-1 treatment
attenuated OM-mediated mitochondrial aspect ratio reductions (Figure 2B) without altering OM-mediated increases in DRP1 protein (Figure 2A). To assess whether the changes in DRP1 and mitochondrial morphology we observed in human SMCs resulted in mitochondrial functional deficits, we utilized the tetramethylrhodamine ethyl ester (TMRE) reagent to measure mitochondrial membrane potential, a general marker of mitochondrial function. TMRE fluorescence was reduced in cells cultured in OM for two weeks, and this decrease was attenuated by DRP1 inhibition (Figure 2C).

Previous studies link DRP1 and Protein Kinase C (PKC), with PKC\(\delta\) inducing DRP1 driven fission activity in SMCs. Therefore we examined PKC in relation to DRP1 changes under calcifying conditions. Immunohistochemistry showed increased PKC\(\delta\) in the same calcified regions of human carotid arteries that stained for DRP1 (Figure 1B). Dexamethasone, a component of OM, increases fatty acid synthesis. Fatty acids increase DRP1 and have been associated with PKC activation. To test whether altered mitochondrial fission observed in OM conditions involved PKC, we measured PKC activity in cells cultured for two weeks in CM and OM. In human SMCs, PKC enzyme activity was elevated in OM with and without the addition of Mdivi-1 (Figure 2D).

DRP1 inhibition attenuated OM induced cytoskeletal changes, TNAP activation, COL1A1 secretion, and human SMC calcification.

The OM-mediated alterations in mitochondrial alignment and aspect ratio that were attenuated by DRP1 inhibition, along with reports of cytoskeleton involvement in DRP1 mediated fission could indicate cytoskeletal alterations. As microtubule stability and PKC activity associate with SMC calcification suppression, we assessed the tubulin and filamentous actin cytoskeleton of human SMCs undergoing osteogenic differentiation. Two-week OM treatment resulted in a modestly diffuse tubulin cytoskeleton and a more apparent actin cytoskeleton; a difference attenuated by Mdivi-1 (Figure 2E). Mdivi-1 increased \(\alpha\)-TUBULIN acetylation, a modification associated with tubulin stability (Online Figure IIB).

We next assessed whether the effects of Mdivi-1 treatment on cytoskeleton reflect the ability of DRP1 inhibition to block the transition of human SMCs to osteoblast-like calcifying SMCs. Intracellular calcium levels analyzed by Fluo-4 did not change in cells treated in OM for two weeks, but slightly increased in both CM and OM conditions with the addition of Mdivi-1 (Figure 3A). DRP1 inhibition reduced OM-induced cellular TNAP mRNA, protein, and activity (Figure 3B-D). OM reduced the calcification inhibitor and TNAP substrate, pyrophosphate (PPI), which was partially restored by DRP1 inhibition in human SMCs (Figure 3E). DRP1 inhibition attenuated OM induced calcification, visualized by Alizarin Red and Osteosense staining (Figure 3F and 3J). To confirm the specificity of DRP1 inhibition in driving the anti-calcification effects of Mdivi-1, we reduced DRP1 in human SMCs with small interfering RNA oligonucleotides (siRNA; DRP1 western blot siRNA confirmation shown in Figure 2A). In agreement with the inhibitor results, DRP1 siRNA attenuated the formation of a mineralized matrix in human SMCs cultured in OM for three weeks (Figure 3F). Mdivi-1 reduced the expression of the TNAP regulator, RUNX2 in human SMCs (Online Figure IIIA). Additionally, DRP1 inhibition attenuated oxidative stress induced mineralization in human SMCs (Figure 3G) treated with a low concentration of hydrogen peroxide (H\(_2\)O\(_2\); 0.3 mmol/L). This concentration of \(\text{H}_2\text{O}_2\) induces SMC calcification without altering cell viability. As DRP1 inhibition did not fully inhibit OM induced TNAP activity, we looked at additional mechanistic pathways through which it may act to inhibit calcification. In our OM experimental conditions, DRP1 inhibition effects on SMC calcification were not mediated by the calcification inhibitors osteoprotegerin and matrix gla protein, ER stress, or oxidative stress without the addition of \(\text{H}_2\text{O}_2\) (Online Figures IIC, IID, IIIB). As calcifying vesicles utilize a collagen matrix to attach and mineralize, we assessed type 1 collagen (COL1A1) content. Mdivi-1 reduced human SMC type 1 collagen secretion from a 7.5-fold induction to a 2.5-fold induction in OM (Figure 3H) without altering COL1A1 mRNA (Figure 3I). Collagen staining using CNA probe validated the increased collagen matrix produced in human OM treated SMCs that was attenuated by DRP1 inhibition (Figure 3J).
To analyze DRP1 in human valve calcification, we performed DRP1 immunostaining on calcified human aortic valves obtained from patients undergoing aortic valve replacement surgery (N=5). DRP1 immunoreactivity was detected in calcified valve tissue (Figure 4A). We next tested the role of DRP1 in human valve calcification using an established cell culture calcification condition, in which primary human valve interstitial cells (VICs) were treated with OM containing inorganic phosphate and L-ascorbic acid for three weeks. DRP1 mRNA was significantly increased in OM (Figure 4B). DRP1 siRNA reduced human VIC calcification (Figure 4C). TNAP mRNA and activity were not induced during VIC calcification, which was reflected by no changes in VIC PPi levels (Figure 4D). Similarly, RUNX2 mRNA was not strongly induced under these experimental conditions (Online Figure IIIC). VIC COL1A1 secretion was increased by ~1.5-fold in OM. This modest induction in VICs was not altered by DRP1 knockdown (Figure 4E). Examination of calcification related transcription factors, MSX2 and SOX9, revealed no changes in MSX2 expression, whereas SOX9 was elevated by DRP1 siRNA in VICs, and by DRP1 inhibition in human SMCs (Online Figure IIIA and IIIC).

Mdivi-1 reduced human PBMC-derived osteoclastogenesis.

We analyzed the in vitro effects of DRP1 inhibition on human vascular osteoclastogenesis through the use of primary human peripheral blood mononuclear cells (PBMCs). PBMCs were cultured in the presence of M-CSF and RANKL to drive osteoclastogenesis. 25 and 50 μmol/L Mdivi-1 reduced tartrate-resistant acid phosphatase (TRAP) activity in PBMCs by 68% and 72%, respectively (Online Figure IVA). 50 μmol/L Mdivi-1 disrupted large actin ring formation in human PBMCs, while actin rings could be seen similar to that observed in untreated cells with 25 μmol/L Mdivi-1, but large multinucleated TRAP-positive cells were still less apparent (Online Figure IVA). Given the similar dose effects observed in the PBMCs to reduce TRAP activity, we used 25 μmol/L Mdivi-1 to test the effects of DRP1 inhibition on human PBMC-derived osteoclast bone resorption. Time course analysis involving adding Mdivi-1 at the start of the two-week osteoclast differentiation procedure, or starting at four days or seven days into the process, showed that Mdivi-1 alters human PBMC-derived osteoclasts through inhibition of differentiation, but not through regulating the bone resorption activity of fully formed osteoclast-like cells (Online Figure IVB).

Mdivi-1 attenuated oxidative stress mediated mouse SMC calcification.

Immunohistochemistry was used to examine DRP1 in calcified Ldlr-deficient, ApoE-deficient, and PCSK9 gain-of-function atherosclerosis mouse models; however, DRP1 was not enriched in calcified mouse plaque (Online Figure I). In agreement, DRP1 protein and PKC activity were not increased in primary mouse SMCs cultured in OM (Online Figure VA and VB), and DRP1 inhibition did not suppress mouse SMC mineralization in OM alone (Online Figure VC). To test if the stress level required to elicit DRP1 changes was not reached in mouse SMC under the examined experimental conditions, we cultured mouse SMCs in OM with the addition of low levels of H2O2 (0.3 mmol/L). H2O2 increased oxidative stress (Online Figure VD) and DRP1 translocation to mitochondria (Figure 5A). Similar to human SMCs in OM, H2O2 addition to mouse SMCs induced Tnap expression (Figure 5B), the production of a calcified collagen-rich matrix (Figure 5C and 5D), and mitochondrial fragmentation (Figure 5E), all of which were attenuated by Mdivi-1 in addition to increasing Sox9 expression (Online Figure IID).

Drp1-deficiency is embryonic lethal in mice, therefore to further examine DRP1 inhibition in a mouse vascular calcification model, we utilized Drp1 heterozygous mice (Drp1+/−), which maintain about 75% of wild type DRP1 protein and are largely indistinguishable from wild type mice. Mice were given a single tail vein injection of murine PCSK9 gain-of-function adeno-associated virus (AAV; which mimics
LDLR-deficiency pathology in mice), fed an atherogenic diet for twenty weeks, and then analyzed in a similar manner to that which we and others have previously reported.42-44 Drp1+/- male mice (N=5) did not have significantly altered body weight, serum triglycerides, or total cholesterol, compared to Drp1+/- male siblings (Figure 6A). Serum total PCSK9 (endogenous + gain-of-function), trended lower but was not significantly reduced in Drp1+/- mice (Figure 6A). Additionally, aortic root Oil Red O, MAC3 (macrophage), and TRAP activity staining was not altered in Drp1+/- aortic roots (Figure 6B). Picrosirius red stain visualized under polarized light (fibrillar collagen accumulation) trended lower, but was not significantly altered in Drp1+/- aortic roots (Figure 6B). In agreement with our primary mouse SMCs in OM without the addition of H2O2 data and the lack of change observed in the mouse vascular DRP1 immunohistochemistry, TNAP activity and vascular calcification measured by Osteosense fluorescence reflectance imaging in mouse aortas were not significantly different in Drp1+/- mice under these experimental conditions (Figure 7).

**DISCUSSION**

We report the following novel findings: 1) DRP1 associates with calcified human cardiovascular tissue and cells; 2) DRP1 inhibition attenuates primary human SMC and VIC calcification; 3) Mdivi-1 reduces profibrotic COL1A1 secretion induced during human SMC osteogenic differentiation; and 4) Mdivi-1 attenuates oxidative stress-mediated human and mouse SMC calcification, in addition to suppressing oxidative stress mediated dysfunction in human osteoblasts. These results support our hypothesis that DRP1 promotes human cardiovascular calcification via regulating osteogenic differentiation. DRP1 associates with cell differentiation, with its inhibition blocking mouse myoblast47 and Drosophila follicle cell48 differentiation. In our calcification model, OM-reduced mitochondrial membrane potential and increased mitochondrial fragmentation could be attenuated by DRP1 inhibition, signifying a role of mitochondrial dynamics in osteogenic differentiation.

Along with its role in osteogenesis, TNAP is generally recognized as a marker of cell differentiation, and DRP1 inhibition reduces TNAP in pluripotent stem cells.

**Osteogenesis**

Along with its role in osteogenesis, TNAP is generally recognized as a marker of cell differentiation, and DRP1 inhibition reduces TNAP in pluripotent stem cells. As DRP1 inhibition reduced TNAP activity in OM treated human SMCs and H2O2 treated mouse SMCs, TNAP reduction is a mechanistic component through which DRP1 regulates the reprogramming network that specifies SMC osteogenic differentiation. In contrast to the reduced TNAP activity we observed in human SMCs, DRP1 inhibition rescues oxidative stress driven TNAP reductions in human bone osteoblast-like cells. Similarly, we found that Mdivi-1 suppressed oxidative stress mediated mineralization loss in primary human osteoblasts. No defects in long bones have been reported in patients with mutations in the DNM1L gene encoding DRP1, and we did not observe changes in mouse bone density in Drp1+/- femurs; suggesting cell type specificity in DRP1 regulation of mineralization. In agreement, the transcriptional program of calcifying SMCs and osteoblasts has been reported to not overlap, suggesting cell type specific mechanistic differences between bone and ectopic calcification, although cell heterogeneity may account for some expression pattern differences. Cell stress can produce cell type specific calcification responses, exemplified by oxidative stress driving SMC
but inhibiting osteoblast mineralization. Mfn2 but not Drp1 is elevated during mouse osteoblast differentiation, and Mfn2 knockdown reduces mitochondrial elongation and osteoblast differentiation. Cells from humans that do not express any wild type DRP1 are resistant to H$_2$O$_2$ induced mitochondrial fragmentation. In agreement, we observed reduced mitochondrial aspect ratio and mineralization in human osteoblasts treated with H$_2$O$_2$, which was suppressed by Mdivi-1. While H$_2$O$_2$ similarly decreased SMC mitochondrial aspect ratio, it increased SMC calcification, which was attenuated by DRP1 inhibition. As such, calcifying SMCs and bone osteoblasts may have different metabolic needs that are better supported by fatty acid oxidation and mitochondrial respiration or glycolysis to supply energy, along with corresponding mitochondrial morphology changes during differentiation.

As our VIC calcification condition did not involve TNAP induction, DRP1 inhibition attenuates cardiovascular cell calcification through additional mechanisms. Along with RUNX2, the transcription factor SOX9 is associated with cardiovascular calcification. Regulation of SOX9 by DRP1 requires further in-depth analysis beyond the scope of the present study. Our data indicate DRP1 inhibition increased SOX9 expression in human SMCs, VICs, and H$_2$O$_2$ treated mouse SMCs. SOX9 inhibits valve calcification. High variability of SOX9 has been reported in non-calcified vessels while calcified carotid lesions contain higher SOX9, making the role of SOX9 in cardiovascular calcification unclear. However, increased SOX9 may act in a compensatory means to attenuate disease progression.

Our previous study associated macrophages with vascular calcification through the production of extracellular vesicles contributing to mineralization. Whether macrophages play an additional role in calcification via mineral resorption by differentiating into osteoclast-like cells in the vasculature, however, remains unclear. Mdivi-1 suppressed PBMC osteoclastic differentiation, but not mineral resorption in fully formed osteoclast in vitro and we did not observe changes in MAC3 staining or TRAP activity in the aorta of heterozygous Drpl-deficient mice. Future studies may address the role of mitochondrial dynamics in monocytes and macrophages during in vivo calcification including conditions of enhanced oxidative stress, as H$_2$O$_2$ enhances osteoclastic differentiation. Collagen is trafficked in vesicles from the endoplasmic reticulum (ER) prior to being secreted from cells, but the molecular mechanisms of how this is accomplished are not clear. As the human SMC increase in OM-induced COL1A1 secretion exceeded the transcriptional increase, one intriguing possibility is that these cells may activate auxiliary mechanisms to increase the rate of collagen secretion involving DRP1. Alternatively, Mdivi-1 may have indirectly altered collagen transport resulting in less COL1A1 secretion in human SMCs undergoing osteogenic differentiation. In rat liver, DRP1 has been observed at the ER, and in fractions containing secretory proteins. In mice in which Drpl is deleted in the liver, VLDL secretion, serum cholesterol, and triglycerides significantly decrease on a high fat diet without altering mitochondrial respiratory activity, liver ATP, or lipid content, further supporting a role of DRP1 in cellular trafficking. In the present study, DRP1 inhibition in SMCs modestly increased intracellular calcium, while blocking extracellular matrix mineralization. Intracellular calcium and calcium binding proteins such as annexins have been associated with extracellular vesicles involved in the calcification process. DRP1 interacts with Annexin A6. Annexin protein may maintain the architectural and functional features of ER exit sites. Whether calcium regulation and annexins participate in DRP1 regulation of protein secretion and calcification requires further exploration. DRP1 is an ER resident protein in mouse β-cells, and dominant-negative Drpl expression helps maintain β-cell ER structure under stressed conditions. Together, these studies suggest a role of DRP1 in ER maintenance, which may explain our observation of decreased OM-induced COL1A1 secretion in Mdivi-1 treated human SMCs.

DRP1 inhibition suppresses vascular disease pathology in diabetic ApoE-deficient mice, and balloon and wire injury rodent models. In contrast, heterozygous Drpl-deficient mice did not have altered vascular pathology in a PCSK9-gain-of-function model, and DRP1 was not elevated in calcified plaque of the two most commonly used atherosclerosis mouse models, ApoE-deficient and Ldlr-deficient mice. We found that attenuation of mouse SMC calcification by Mdivi-1 required the addition of oxidative stress.
stress to the osteogenic differentiation condition. As oxidative stress is elevated in diabetic and injury models, DRP1 inhibition may require the presence of certain stress levels in order to attenuate disease pathology in murine cardiovascular models. Oxidized low density lipoprotein enhances SMC but suppresses osteoblast differentiation, increases cellular oxidative stress, and induces DRP1 mediated mitochondrial fission in SMCs. A recent study found that treating rat SMCs with the antioxidant flavonoid Quercetin reduced DRP1 abundance and phosphate induced calcification in an oxidative stress dependent manner in vitro, along with suppressing calcification in adenine fed rats, a model of chronic kidney disease. While DRP1 abundance and direct inhibition of DRP1 remain to be examined in chronic kidney disease, mitochondrial diameter is increased in the kidneys of diabetic mice, and this increase is attenuated by dominant-negative DRP1. The diabetes medication metformin suppresses diabetes-accelerated atherosclerosis in mice through DRP1 inhibition, and attenuates progression of arterial calcification in HIV-infected metabolic syndrome patients. Given the connection between metabolic disease and cardiovascular calcification, conditions with enhanced oxidative stress, like diabetes and chronic kidney disease, may provide clinically relevant in vivo models in which to further assess DRP1 inhibition effects on cardiovascular calcification. Humans heterozygous for DRP1 deletion or missense mutations do not have associated medical issues, and Mdivi-1 does not produce organ toxicity in rats; however, several concerns need to be addressed before assessing DRP1 inhibition in a clinical setting, particularly under prolonged treatment conditions. Despite high unmet clinical needs of cardiovascular calcification, the underlying mechanisms remain obscure. Our current study identifies DRP1 as a novel regulator of cellular fibrocalcific response, which may lead to further research supporting the development of new therapies to combat cardiovascular calcification, a global health burden.

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DISCLOSURES
None.
REFERENCES


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FIGURE LEGENDS

Figure 1. DRP1 was enriched in calcified regions of human carotid arteries. Representative human carotid artery tissue immunohistochemistry for (A) DRP1, (B) PKCδ, and adjacent sections for DRP1, CD68, and HHF-35 (smooth muscle cell marker) in calcified tissue (C); N=5 donors. (D) Representative immunofluorescence images showing DRP1 staining in both αSMA and CD68 positive cells in calcified areas of human carotid artery; N=5 donors. (E) Representative electron microscopy images of human carotid artery smooth muscle cells (SMCs) in non-calcified and calcified tissue regions. Arrows indicate examples of mitochondria. Relative quantification of SMC mitochondrial area (N=32-41 mitochondria from 3 donors); error bars indicate SEM; *** P<0.001.

Figure 2. DRP1 and mitochondrial dysfunction increased in calcifying human SMCs. (A) DRP1 mRNA and protein from human smooth muscle cells (SMCs) cultured in control (CM) or osteogenic media (OM) for two weeks with vehicle (0.01% DMSO), Mdivi-1 (50 μmol/L), control siRNA (CONsi), or DRP1 siRNA (DRP1si). (B) Representative MitoTracker Red (mitochondria) human SMC processed binary confocal images from human SMCs cultured in CM or OM for two weeks. Red asterix indicates the location of a representative cell with mitochondrial alignment shown in the corresponding images below (Mitochondria color coded by major axis orientation). Quantified mitochondrial aspect ratio and alignment from SMCs cultured in CM or OM with vehicle or Mdivi-1 treatment for two weeks. (C) Mitochondrial membrane potential assayed by TMRE fluorescence and (D) PKC activity in human SMCs treated for two weeks. (E) Representative confocal microscopy images of DAPI, α-TUBULIN, and phalloidin from human SMCs treated for two weeks. Error bars indicate STDEV; ns= not significant. * P<0.05, **<0.01, ****<0.0001; N=3-4 donors.

Figure 3. DRP1 inhibition attenuated human SMC osteogenic media-induced fibrocalcific response. (A) Live cell intracellular calcium concentrations, and (B) TnAP mRNA, (C) protein, and (D) relative enzyme activity from human coronary smooth muscle cells (SMCs) cultured in control media (CM) or osteogenic media (OM) for two weeks with DMSO vehicle (0.01%) or Mdivi-1 (50 μmol/L). (E) Relative media pyrophosphate (PPi) from human coronary SMCs treated for two weeks. (F) Representative Alizarin Red stain image and quantification from human coronary artery SMCs treated with Mdivi-1 or DRP1 siRNA for three weeks. (G) Representative Alizarin Red stain and quantification for human aortic SMCs treated for three weeks in OM with H2O2 (0.3 mmol/L), with or without Mdivi-1. (H) Human coronary artery SMC media COL1A1 ELISA from cells treated for two weeks. (I) COL1A1 mRNA from human coronary artery SMCs treated for two weeks. (J) Representative Osteosense (red; calcification) and CNA immunofluorescence (green; collagen) from human coronary artery SMCs treated for three weeks. N=3-6 donors; ns not significant, * P<0.05, **<0.01, ***<0.001, ****<0.0001; error bars indicate STDEV.

Figure 4. DRP1 knockdown attenuated human valve interstitial cell calcification. (A) Representative DRP1 immunohistochemistry image from calcified human aortic valve and adjacent section control (secondary antibody only) (N=5 donors). (B) DRP1 mRNA in primary human valve interstitial cells (VICs) treated in control media (CM) or osteogenic media (OM) with control or DRP1 siRNA for two weeks. (C) Representative Alizarin Red staining and quantification from human VICs treated for three weeks. (D) Media pyrophosphate and (E) media COL1A1 ELISA from VICs treated for two weeks. N=3 donors; ns= not significant, * P<0.05, **<0.01, ***<0.001, ****<0.0001; error bars indicate STDEV.

Figure 5. Mdivi-1 attenuated H2O2 induced calcification in mouse SMCs. (A) Western blot analysis of DRP1 translocation to the mitochondria five hours post-treatment in control (CM) and osteogenic media (OM) with H2O2 (0.3 mmol/L), with DMSO vehicle (0.01%) or Mdivi-1 (50 μmol/L); TOMM20 served as mitochondrial fraction loading control. (B) TnAP mRNA from mouse SMCs cultured for two weeks. (C) Alizarin Red stain with quantification, and (D) Osteosense (red; calcification) and CNA (green; collagen) immunofluorescence from mouse aortic SMCs treated for three weeks. (E) MitoTracker Red stain.
immunofluorescence (white boxes indicate higher magnification area of corresponding lower panels) and mitochondrial aspect ratio quantification. \( N=3; \) ns=not significant, * \( P<0.05, \) **\( P<0.01, \) ***\( P<0.001, \) ****\( P<0.0001; \) error bars indicate STDEV.

**Figure 6.** Drp1\(^{+/−}\) did not significantly alter atherosclerosis pathology in a PCSK9 gain-of-function AAV mouse model. (A) Body weight and serum triglycerides, total cholesterol, and PCSK9 from mice twenty weeks post-PCSK9 gain-of-function AAV tail vein injection and maintained on an atherogenic diet. (B) Representative images of aortic root Oil Red O, MAC3 (macrophage), TRAP activity, and Picrosirius Red staining. \( N=5 \) C57Bl/6;129 male mice per group, with at least two sections per mouse analyzed; ns=not significant; error bars indicate STDEV.

**Figure 7.** Drp1\(^{+/−}\) did not significantly alter vascular calcification pathology in a PCSK9 gain-of-function AAV mouse model. (A) TNAP activity representative images and quantification. (B) Calcification in intact mouse aortas (fluorescence reflectance imaging with Osteosense) 20 weeks post-PCSK9 gain-of-function AAV tail vein injection and maintained on an atherogenic diet. \( N=5 \) C57Bl/6;129 male mice per group, with at least two sections per mouse analyzed for immunohistochemistry; ns=not significant; error bars indicate STDEV.

**Figure 8.** DRP1 inhibition did not reduce bone mineralization. (A) Representative femur cortical and trabecular bone micro-CT images and trabecular thickness, trabecular number, trabecular spacing, cortical thickness, and bone volume/trabecular volume quantification (\( N=5 \) C57Bl/6;129 male mice/group). (B) Two week cultured MitoTracker Red stain (white boxes indicate higher magnification area of corresponding images below) and mitochondrial aspect ratio quantification, and (C) three-week cultured Alizarin Red staining and quantification from primary human bone osteoblasts treated in control media (CM) or osteogenic media (OM) with H2O2 (0.3 mmol/L), and with DMSO vehicle (0.01%) or Mdivi-1 (50 \( \mu \)mol/L); \( N=3; \) ns= not significant, * \( P<0.05, \) ***\( P<0.001; \) error bars indicate STDEV.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Dynamin-related protein 1 (DRP1) regulates mitochondrial fission.

- Mitochondrial morphology changes during cell differentiation, and DRP1 has been shown to promote the differentiation of some cell types.

What New Information Does This Article Contribute?

- A novel association of DRP1 to cardiovascular calcification, with increased DRP1 observed in calcified human cardiovascular tissue and cells.

- DRP1 inhibition attenuated primary human smooth muscle cell and valve interstitial cell calcification, inhibited oxidative stress-mediated smooth muscle cell calcification, and prevented oxidative stress-mediated dysfunction in bone osteoblasts.

DRP1 is a promising therapeutic target for multiple diseases; however, the role of DRP1 in cardiovascular disease is not clearly defined. Here we show that DRP1 associates with calcified human plaques and regulates osteogenic differentiation of cardiovascular cells. Additionally, we report a novel function of DRP1 in regulating osteogenic differentiation-induced type 1 collagen secretion. These results support a role of mitochondrial dynamics in the opposite mineralization response of vascular and bone cells to oxidative stress, and better define the role of DRP1 and mitochondrial dynamics in cardiovascular disease.
FIGURE 1

A. Calcified human carotid artery

B. PKCδ

C. DRP1, CD68, HHF-35

D. Calcified region

E. Non-calcified region

Mitochondrial area

Relative area (μm²)

0.0

0.5 μm

0.5 μm

Calcified region
**FIGURE 2**

**A**

- **DRP1 mRNA**
  - Relative expression (Fold Change, DCt)
  - CM+DMSO, OM+DMSO, CM+Mdivi-1, OM+Mdivi-1

- **DRP1 Protein**
  - Relative abundance (normalized to β-Actin)
  - CM+DMSO, OM+DMSO, CM+Mdivi-1, OM+Mdivi-1

**B**

- **CM+DMSO**, **OM+DMSO**, **CM+Mdivi-1**, **OM+Mdivi-1**

**C**

- **TMRE**
  - Relative TMRE (fluorescence/mg protein)
  - CM+DMSO, OM+DMSO, CM+Mdivi-1, OM+Mdivi-1

**D**

- **PKC activity**
  - Relative kinase activity
  - CM+DMSO, OM+DMSO, CM+Mdivi-1, OM+Mdivi-1

**E**

- **MERGE**, **DAPI**, **α-TUBULIN**, **PHALLOIDIN**
  - CM+DMSO, OM+DMSO, CM+Mdivi-1, OM+Mdivi-1
FIGURE 3

A. Intracellular calcium

B. TNAP mRNA

C. TNAP protein

D. TNAP activity

E. Media pyrophosphate

F. Human SMC
   Alizarin Red (calcification)

G. Human SMC
   Alizarin Red (calcification)

H. Alizarin Red

I. COL1A1 mRNA

J. Human SMC
   Osteosense (calcification) and CNA probe (collagen)
A

Calcified human aortic valve

DRP1 immunohistochemistry

secondary only control immunohistochemistry

B

C

Human VIC Alizarin Red
(calcification)

D

E

VIC media pyrophosphate

VIC media COL1A1 protein
**FIGURE 6**

### A

**Body weight**

<table>
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<th></th>
<th>Drp1&lt;sup&gt;+&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Drp1&lt;sup&gt;+/−&lt;/sup&gt;</th>
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<tr>
<td>Drp1&lt;sup&gt;+/−&lt;/sup&gt;</td>
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<td>Drp1&lt;sup&gt;+/−&lt;/sup&gt;</td>
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</tbody>
</table>

### B

**Drp1<sup>+<sup>+</sup>/**

- **Oil Red O**
- **MAC3**
- **TRAP activity**
- **Picrosirius Red**

**Drp1<sup>+/−</sup>**

- **Oil Red O**
- **MAC3**
- **TRAP**
- **Picrosirius Red**
FIGURE 7

A

<table>
<thead>
<tr>
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<tr>
<td>% (TNAP positive root)</td>
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<tr>
<td>Drp1+/+</td>
<td>2.5</td>
</tr>
<tr>
<td>Drp1+/+</td>
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</tr>
<tr>
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</table>

**TNAP**

**Drp1+/+**

**Drp1+/+**

**Drp1+/+**

**B**

**Drp1+/+**

**Drp1+/+**

**Drp1+/+**

**Aorta Osteogenesis**

<table>
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<th>Osteosecreton positive area (%)</th>
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</tr>
<tr>
<td>Drp1+/+</td>
<td>ns</td>
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**ns**
Dynamin-Related Protein 1 Inhibition Attenuates Cardiovascular Calcification in the Presence of Oxidative Stress
Maximillian Rogers, Natalia Maldonado-Martinez, Joshua D Hutcheson, Claudia Goettsch, Shinji Goto, Iwao Yamada, Tyler Faits, Hiromi Sesaki, Masanori Aikawa and Elena Aikawa

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Supplemental Material

Materials and Methods

**Human tissue**
Atherosclerotic carotid artery samples were obtained from endarterectomy patients (Brigham and Women’s Hospital Institutional Review Board protocol #1999P001348), embedded in optimum cutting temperature compound and stored at -80°C until use. Autopsy carotid artery samples were obtained from Brigham and Women’s Hospital (Institutional Review Board protocol #2013P002517/BWH), cut and then stored at -80°C until use. Human aortic valve tissue was obtained from patients undergoing valve replacement (Institutional Review Board protocol #2011P001703), embedded and stored at -80°C until use.

**Primary cell culture**
Primary human coronary artery and aortic smooth muscle cells were obtained from Promocell (Heidelberg, Germany) and expanded in SMC Growth Medium 2 (Promocell) supplemented with epidermal growth factor (0.5 ng/mL), insulin (5 μg/mL) basic fibroblast growth factor-B (2 ng/mL), and 5% fetal bovine serum. Cells were cultured at 37°C (5% CO2, 90% humidity) and used between passages 3 and 10. Cells from at least three human donors were used. Primary C57Bl/6 mouse aorta SMCs (CellBiologics, Chicago, IL) were cultured using the same methods and media as primary human SMCs. Both human (PromoCell) and mouse (CellBiologics) primary SMCs were isolated via collagenase tissue digestion. SMC identity was confirmed by αSMA FACS analysis using a BD FACS Aria™ IIu flow cytometer (BD Biosciences, Franklin Lakes, NJ) and anti-α-smooth muscle-cy3 antibody (#C6198, Sigma). Primary human VICs were obtained from isolated human aortic valve tissue in a similar manner as reported.

**PCSK9 gain-of-function AAV**
PCSK9 gain-of-function AAV injections were performed as previously described. Briefly 10-week-old male mice were given a single tail vein injection of recombinant murine PCSK9 AAV (pAAV/D377Y-mPCSK9; 1X10^{11} vector genome copies/injection diluted in 200μL sterile saline solution). AAV was produced at the University of North Carolina Vector Core (Chapel Hill, NC) using the murine PCSK9 gain-of-function plasmid generated by Bjørklund et al. and obtained from Addgene (#58376; Cambridge, MA).

**Mice**
C57Bl/6;129 heterozygous Drp1^{−/−} mice have been previously described. Age-matched sibling male wild type (Drp1^{+/+}) and heterozygous (Drp1^{−/+}) mice were used for experiments. Male C57Bl/6 wild type mice (#000664; Jackson Laboratory), Apolipoprotein E-deficient (ApoE^{−/−}) mice (#002052; Jackson Laboratory, Bar Harbor, ME), Ldlr-deficient mice (#002207; Jackson Laboratory), and PCSK9 gain-of-function AAV injected mice were fed an atherogenic high-fat, high-cholesterol diet (21% fat and 1.25% cholesterol, Research Diets D12108C, New Brunswick, NJ, USA) for 18-20 weeks. All Drp1^{+/+} and Drp1^{−/−} mice used in experiments were maintained on atherogenic diet for 20 weeks. Following 18-20 weeks on the atherogenic diet, mice were carbon dioxide euthanized. Blood was collected by cardiac puncture, and serum was isolated by spinning samples at 9,000 RPM for 15 minutes, and stored at -80°C until use. Tissues for histology were embedded in optimum cutting temperature compound and stored at -
80°C until use. All animal procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (protocol #010-2013).

**Immunohistochemistry and immunofluorescence**

Tissues were cut into slices with 7 μm thickness, and cryosections were fixed in acetone or 4% paraformaldehyde. Following blocking in 4% serum, sections were incubated in primary antibodies: DRP1 for human IHC [1:50, Abcam #ab56788, Cambridge, MA]; as the DRP1 antibody used for human IHC was made in mouse, a different monoclonal antibody made against mouse DRP1 in rabbit was used for mouse DRP1 IHC [1:250, Abcam #ab184247]; DRP1 for IF [1:50, Cell Signaling Technology #8570, Danvers, MA]; CD68 for IHC [1:700, Dako #M0876, Carpinteria, CA]; CD68 for IF [1:200, Dako]; HHF-35 [1:35, Enzo Life Sciences #ENZ-C34931, Farmingdale, NY]; α-SMA [1:200, Dako #M0851; for IHC used with secondary antibody #B0287 from Sigma, St. Louis, MO]; PKCδ [1:50, Santa Cruz #sc-937, Dallas, TX]; MAC3 [1:900, BD Biosciences #550292, San Jose, CA], and then incubated with biotin-labeled secondary antibody (Vector Laboratories, Burlingame, CA), followed by incubation with streptavidin-peroxidase (KPL, Gaithersburg, MD), and then incubation in AEC solution (Dako). Oxidative stress immunofluorescence analysis was performed using the Cellular Reactive Oxygen Species Deep Red Detection Assay kit according to manufacturer’s protocol (Abcam). Tartrate-resistant acid phosphatase (TRAP) was stained using the TRAP staining kit (B-Bridge International, Cupertino, CA). TNAP activity was stained using the VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA). Neutral lipids were detected by Oil Red O staining. Collagen was stained using 0.1% Picrosirius Red (pH=2; Polysciences, Warrington, PA). Stains were quantified according to the methods previously described in Yabusaki et al., all values were obtained in a blinded analysis. For immunofluorescence of smooth muscle cells, cells were grown on 0.1% gelatin coated Lab-Tek II chambered cover glass #1.5 borosilicate slides (Lab-Tek, Rochester, NY), washed with PBS and then fixed in 4% paraformaldehyde for 15 minutes, followed by permeabilization using 0.3% Triton X-100 for 5 minutes, washed, and then blocked with 1% BAS in PBS for 30 minutes. Calcification immunofluorescence staining was performed by incubating cells with Osteosense-680 EX [1:100; PerkinElmer, Boston, MA] overnight at 37°C. Collagen was stained with the CNA collagen probe (provided by Carlijn Bouten) similar to how we have previously described, by incubating cells at 37°C with CNA probe (1:50) for 1 hour prior to fixation. Actin was stained using CytoPainter Phalloidin iFluor 555 Reagent [2 μl phalloidin in 2000 μl PBS with 1% BSA; Abcam], with a 60 minute incubation at room temperature. Tubulin was imaged by incubating slides with anti-α-tubulin [1:200, Abcam #ab15246] for 2 hours at room temperature, followed by incubation with Alexa Fluor 488-labelled secondary antibody [1:200; Life Technologies, Carlsbad, CA]. Nuclear staining was performed with DAPI (Life Technologies). Prior to visualization 200 μl PBS was added to each well. Slides were examined using the confocal microscope A1 (Nikon Instruments Inc., Melville, NY), and all images were processed with Elements 3.20 software (Nikon Instruments Inc.).

**Osteogenic differentiation**

Fully confluent control smooth muscle cells (SMCs) were cultured for a total of 21 days in DMEM containing 4.5 g/L glucose and L-glutamine (Lonza, Walkersville, MD), supplemented with 10% FBS and 1% penicillin-streptomycin (termed control media, CM). To induce osteogenic differentiation of SMCs, fully confluent cells were cultured for a total of 21 days in CM with the following additions: 10 nmol/L dexamethasone, 10 nmol/L β-glycerol phosphate, and 100 μmol/L L-ascorbic acid 2-phosphate (termed osteogenic media, OM). Oxidative stress was induced by the addition of 0.3 mM H2O2 with each media change. For SMC DRP1 inhibition experiments, Mdivi-1 (50 or 25 μmol/L) in dimethyl sulfoxide (DMSO; final DMSO concentration of 0.01%) or DMSO vehicle control (0.01%) was added to cell culture media. Mdivi-1 was solubilized in media prior to changing cell culture media by repeated hard vortexing for several minutes and warming media to 37°C until Mdivi-1 was fully solubilized.
Primary human VICs were cultured with some differences in the calcification media compared to that used for SMCs, based on the VIC calcification method used by Bouchareb et al. Briefly, VICs were cultured for a total of 21 days in CM (DMEM containing 4.5g/L glucose and L-glutamine, supplemented with 5% FBS and 1% penicillin-streptomycin). To induce osteogenic differentiation of VICs, fully confluent cells were cultured for a total of 21 days in CM with the following additions: 2 mmol/L NaH₂PO₄ and 50 μg/mL L-ascorbic, termed osteogenic media (OM). Media was changed every three days.

**RNA analysis**

TriZol (Life Technologies, Grand Island, NY) was used to isolate RNA from cultured cells. QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to perform reverse transcription on total RNA. cDNA was generated using the Quanta qScript cDNA Synthesis Kit (Bioscience Inc, Gaithersburg, MD). mRNA expression was quantified by TaqMan real-time PCR (Life Technologies) with the following probes: Hs02758991_g1 (human GAPDH); Mm99999915_g1 (mouse Gapdh); Hs00247147_m1 (human DRP1); Mm01342903_m1 (mouse Drp1); Hs01029144 (human TNAP); Mm00475834_m1 (mouse Tnap); Hs00164004_m1 (human COL1A1); Hs01001343_g1 (human SOX9); Mm00448840_m1 (mouse Sox9); Hs00751239_s1 (human MSX2); Hs00231692_m1 (human RUNX2). Expression levels were normalized to GAPDH. Results were quantified using the ΔΔCT method.

**RNA interference**

RNA knockdown was performed by using 20 nmol/L validated DRP1 siRNA: 4390824 (DRP1; ThermoFisher), 4390843 (negative control; ThermoFisher). Transfection was performed using Lipofectamine RNAiMAX (ThermoFisher). siRNA was added at the beginning of each experiment, and with each media change every three days until sample collection.

**Protein analysis**

Cells and tissues were lysed in RIPA buffer (Thermo Scientific, Rockford, IL) containing Halt combined protease and phosphatase inhibitor cocktails (Thermo Scientific). Protein content was determined by the Pierce BCA assay (Thermo Scientific). Protein lysate was loaded onto 4-15% gels (Bio-Rad, Hercules, CA) in SDS loading buffer (Boston BioProducts, Ashland, MA). Primary antibodies used for Western blot analysis included: DRP1 (1:1,000; Abcam and Cell Signaling Technology), β-actin (1:5,000, Novus, Littleton, CO), CHOP (1:1000, Cell Signaling Technology), PERK (1:1000, Cell Signaling), α-tubulin (1:1000, Abcam), acetyl-alpha-tubulin (1:1000, Cell Signaling Technology), TOMM20 (1:1000, Abcam). DRP1 translocation to the mitochondria in response to H₂O₂ treatment was assessed by cell fraction and Western blot analysis. Five hours post-treatment cells were lysed in sucrose buffer (0.25mol/L sucrose, 1mmol/L EDTA, 10 mmol/L Tris-HCL, pH 7.4, and Halt inhibitor cocktail), and spun at 1,000g to remove nuclei and cell debris. Supernatant was then transfer to a new tube and spun at 15,000g for 15 minutes. The supernatant (containing the cytosolic and microsomal fraction) was collected. The pellet (containing the mitochondrial fraction) was suspended in lysis buffer and both fractions were then loaded on 4-15% gels and analyzed by Western blot. Oxidative stress was measured by use of the Oxyblot Kit (EMD Millipore). COL1A1 ELISAs (human SMC media 1:2000 dilution, human VIC media 1:1000 dilution, R&D Systems, Minneapolis, MN), MGP ELISA (Lifespan Bioscience, Inc.), OPG (R&D Systems) were all performed according to manufacturer’s instructions.

**Mitochondrial aspect ratio and TMRE**

Following smooth muscle cell mitochondria staining with 50 nmol/L MitoTracker Red (ThermoFisher; 15 minute incubation at 37°C) images were acquired by confocal microscopy (Nikon Instruments Inc.) using a 40X immersion objective and processed for quantification using an in-house code in Matlab (Mathworks, Natick, MA) adapted from Lihavainen et al. First, a high pass filter and a median filter were
applied to remove background and noise. The preprocessed images were then binarized with a common threshold to allow segmentation of the mitochondria and the binarized images were used to select single cells. To obtain a measure of the shape each mitochondrion was approximated to an ellipse. The major and minor axes were used to calculate the aspect ratio. Mitochondrial aspect ratios were additionally assessed using MitoTracker Red images in conjunction with NIH ImageJ software. The orientation of the major axis was used to assess mitochondrial alignment and color code individual mitochondrion to assess mitochondrial alignment. Basic mitochondria function was assessed using the tetramethylrhodamine ethyl ester (TMRE) Mitochondria Membrane Potential Assay Kit (Abcam), with no TMRE added samples serving as negative controls. Smooth muscle cells were grown on 96-well plates and live cell analysis for TMRE was performed by a colorimetric method using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

**Protein Kinase C activity**
Protein kinase C activity was assessed by lysing fully confluent smooth muscle cells cultured for 14 days in RIPA buffer containing EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysate was analyzed using the PKC Kinase Enzyme Activity Kit (Enzo Life Sciences, Farmingdale, NY), with values made relative to the control media with vehicle (0.01% DMSO) group.

**Intracellular calcium quantification**
Cells were cultured in 96-well black walled plates for 14 days and live cell intracellular calcium was determined with the Fluo-4 Direct Calcium Assay (ThermoFisher) according to manufacturer’s protocol using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

**TNAP, Alizarin Red, PPI, and aorta Osteosense analysis**
Tissue non-specific alkaline phosphatase (TNAP) activity was analyzed using whole cell lysates and the TNAP enzyme activity assay (BioVision, Inc., Milpitas, CA). Matrix calcium deposition was analyzed by the use of Alizarin Red staining. Briefly cells were washed in PBS then fixed in 4% paraformaldehyde for 30 minutes, and then washed with distilled water. Cells were stained with freshly prepared and filtered Alizarin Red S (Sigma), pH 4.2, with excess stain being removed with four subsequent distilled water washes. Images were taken using a Nikon camera. Alizarin Red stain was quantified by extracting the stain from cells using 100 mmol/L cetylpyridinium chloride with gentle shaking at room temperature for 30 minutes, and measuring the absorbance at 540nm. Pyrophosphate (PPI) was measured using conditioned media collected from cells after 14 days in culture using the High Sensitivity Pyrophosphate Assay Kit (Sigma Aldrich). Calcification in the mouse aorta was analyzed via Osteosense (100 μL) tail vein injections 24 hours prior to euthanization and imaging. Aortas were perfused with saline, dissected and imaged using fluorescent reflection imaging (Image Station 4000 MM, Eastman Kodak, New Haven, CT). Imaging was performed using excitation/emission filter sets of 630 nm/700 nm with a f-stop setting of 2.4, the field of view set to 83.96 mm, the focal plane set at 7.61 mm, and an exposure time of 180 seconds. A custom MATLAB script was used to calculate the positive stained regions, as previously described in detail3.

**Electron microscopy**
Electron microscopy was performed at the Beth Israel Deaconess Medical Center Electron Microscopy Core and additionally at the Massachusetts General Hospital Program in Membrane Biology Electron Microscopy Core. Briefly, tissue was fixed in modified Karnovsky’s fixative (2.5% glutaraldehyde, 2% paraformaldehyde, in 0.1 mol/L cacodylate buffer, pH 7.4), and post-fixed in 1% osmium tetroxide, dehydrated, and then embedded in Epon resin. Grids were imaged on a JEOL 1400 TEM equipped with a side mount Gatan Orius SC1000 digital camera. EM images were quantified using Nikon NIS-Elements
AR software. A region of interest was drawn around each mitochondrion using the software regions of interest (ROI) tool. The area of the ROI (μm²) was calculated with the software ROI Statistics tool.

Serum analysis
Serum total cholesterol and triglyceride levels were quantified using the Total Cholesterol E Kit (Wako Chemicals, Richmond VA) and the LabAssay Triglyceride Kit (Wako Chemicals), with colorimetric analysis performed using a SpectraMax M5 plate reader. PCSK9 was quantified using the mouse PCSK9 Quantikine ELISA Kit (1:20,000 dilution used for PCSK9 gain-of-function AAV injected mice; R&D Systems) according to manufacturer’s protocol.

Bone micro-CT and bone marrow cell isolation/differentiation
Femur bones were collected from male Drp1+/+ and Drp1−/− mice following 20 weeks on atherogenic diet (1.25% cholesterol, D12108C, Research Diets Inc.). Bones were stored in 70% ethanol and kept at 4°C until processing. Blinded micro-CT analysis was performed by the Harvard School of Dental Medicine micro-CT Core facility using X-ray microtomography. Primary human femoral bone osteoblast cells were plated at a 100% confluency and cultured in control media (DMEM+10%FBS+1% pen strep), or the same osteogenic media (OM) used to drive mineralization in smooth muscle cells with the slight modification of using DMEM containing 1g/L glucose (ThermoFisher,Waltham, MA). DRP1 was inhibited by the addition of 50 μmol/L Mdivi-1. Oxidative stress was induced by the addition of 0.3 mmol/L H2O2 with each media change. Media was changed every three days, with Alizarin Red staining analyzed after three weeks in culture in the same manner as performed with the SMCs.

Osteoclast differentiation and analysis
Human peripheral blood mononuclear cells (PBMCs) were isolated from commercially obtained buffy coat (Research Blood Components, Brighton, MA), and differentiated to osteoclasts using 50 ng/mL M-CSF for one day, followed by culturing with M-CSF and 50 ng/mL human RANKL(PeproTech, Rocky Hill, NJ) for 14 days. RANKL-treated cells were analyzed for TRAP activity and staining (B-Bridge International, Cupertino, CA). TRAP stained cells were imaged using an Eclipse TS100 microscope (Nikon) with SPOT idea digital camera and SPOT Basic Software (SPOT Imaging Solutions, Sterling Heights, MI). Bone resorption was analyzed by using Osteo Assay Surface 24-well plates (Corning, Corning, NY) plating PBMCs at 4X10⁴ cells/well, and following the described osteoclast differentiation method. Pit formation was assessed under a light microscope and quantified based on binarized images using Matlab (Mathworks).

Statistical analysis
Data was analyzed using t-test or ANOVA with post hoc tests where appropriate, using PRISM software (GraphPad, San Diego, CA). P-values less than 0.05 were considered significant.

Supplementary References


Online Figure I. DRP1 was not enriched in calcified aorta of commonly used atherosclerosis mouse models. (A) Representative DRP1 immunohistochemistry images of calcified aortic arches from wild type, ApoE<sup>−/−</sup>, Ldlr<sup>−/−</sup>, and PCSK9 gain-of-function AAV mice maintained on an atherogenic diet for 18-20 weeks; arrows indicate DRP1 in vascular wall cells (red reaction product) (N=5). (B) Representative DRP1 staining and quantification in non-calcified and calcified tissue regions of human carotid arteries; additional data shown in Figure 1A (N=5 donors). **** P <0.0001; error bars indicate SEM.
Online Figure II. Mdivi-1 increased acetylated α-TUBULIN in human SMCs. (A) Confirmation of human and mouse SMC identity by αSMA FACS analysis. (B) Acetylated α-TUBULIN protein from human SMCs treated with control (CM) or osteogenic media (OM) with Mdivi-1 (50 μmol/L) or DMSO vehicle (0.01%) for two weeks. (C) Representative PERK and CHOP western blots (below detection) and (D) Oxyblot (oxidative stress) for human SMCs treated for two weeks with (+) or without (-) vehicle (0.01% DMSO), Mdivi-1 (50 μmol/L), control siRNA (CONsi) or DRP1 siRNA (DRP1si). N=3 donors for human SMCs, and 3 experiments for mouse SMCs; ns= not significant, ** P <0.01, *** <0.001; error bars indicate STDEV.
Online Figure III. DRP1 inhibition increased SOX9 expression in human SMCs, VICs and H2O2 treated mouse SMCs. (A) SOX9, MSX2, RUNX2 mRNA, and (B) cell culture media OPG and MGP ELISA from human SMCs cultured for two weeks in control (CM) or osteogenic media (OM) with Mdivi-1 (50 μmol/L) or DMSO vehicle (0.01%). (C) SOX9, MSX2, and RUNX2 mRNA from human VICs treated with control siRNA (CONsiRNA) or DRP1 siRNA (DRP1siRNA) for two weeks. (D) Sox9 mRNA from mouse SMCs cultured for two weeks in CM or OM with H2O2 (0.3 mmol/L), with or without Mdivi-1 (50 μmol/L), N=6 donors for human SMCs, 3 donors for VICs, and 3 experiments for mouse SMCs; ns= not significant, * P <0.05, ** <0.01, *** <0.001; error bars indicate STDEV.
Online Figure IV. Mdivi-1 reduced human PBMC osteoclastogenesis. (A) Representative actin and TRAP staining images, and TRAP activity quantification for human peripheral blood mononuclear cells (PBMCs) from control (no RANKL), and cells differentiated into osteoclasts-like cells with the addition of RANKL or RANKL with 25 or 50 μmol/L Mdivi-1; N=6. (B) Representative bone resorption (light gray color) images of human PBMCs differentiated into osteoclasts with RANKL or RANKL with 25 μmol/L Mdivi-1 added at the beginning of the differentiation process or at days four or seven; N=6. * P <0.05, ** <0.01, ***<0.001, ****<0.0001; error bars indicate STDEV.
Online Figure V. Mdivi-1 did not alter primary C57Bl/6 mouse aortic SMC osteogenic media-induced calcification without the addition of oxidative stress. (A) DRP1 protein from C57Bl/6 aortic SMCs cultured for two weeks in control media (CM) or osteogenic media (OM) with vehicle (0.01% DMSO) or Mdivi-1 (50 μmol/L). (B) PKC activity from C57Bl/6 SMCs treated for two weeks. (C) Representative Osteosense (red; calcification) and CNA probe (green; collagen) immunofluorescence of C57Bl/6 SMCs treated for three weeks. (D) Reactive oxygen species (ROS) immunofluorescence from C57Bl/6 SMCs treated for two weeks with or without H$_2$O$_2$ (0.3 mmol/L) and Mdivi-1 (50 μmol/L). N= 3; error bars indicate STDEV; ns= not significant, * P <0.05.
Online Figure VI. Osteogenic media induced calcification in human bone osteoblasts. (A) Alizarin Red staining and (B) Osteosense staining from primary human bone osteoblasts treated in control media (CM) or osteogenic media (OM) with DMSO vehicle (0.01%) or Mdivi-1 (50 μmol/L) for two weeks; N=3; ns= not significant, *** P <0.001; error bars indicate STDEV.