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Metformin protects against vascular calcification through the selective degradation of Runx2 by the p62 autophagy receptor

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Abstract

Vascular calcification is associated with aging, type 2 diabetes, and atherosclerosis, and increases the risk of cardiovascular morbidity and mortality. It is an active, highly regulated process that resembles physiological bone formation. It has previously been established that pharmacological doses of metformin alleviate arterial calcification through adenosine monophosphate-activated protein kinase (AMPK)-activated autophagy, however the specific pathway remains elusive. In the present study we hypothesized that metformin protects against arterial calcification through the direct autophagic degradation of runt-related transcription factor 2 (Runx2). Calcification was blunted in vascular smooth muscle cells (VSMCs) by metformin in a dose-dependent manner (0.5–1.5 mM) compared to control cells ($p < 0.01$). VSMCs cultured under high-phosphate (Pi) conditions in the presence of metformin (1 mM) showed a significant increase in LC3 puncta following bafilomycin-A1 (Baf-A; 5 nM) treatment compared to control cells ($p < 0.001$). Furthermore, reduced expression of Runx2 was observed in the nuclei of metformin-treated calcifying VSMCs ($p < 0.0001$). Evaluation of the functional role of autophagy through *Atg3* knockdown in VSMCs showed aggravated Pi-induced calcification ($p < 0.0001$), failure to induce autophagy (punctate LC3) ($p < 0.001$) and increased nuclear Runx2 expression ($p < 0.0001$) in VSMCs cultured under high Pi conditions in the presence of metformin (1 mM). Mechanistic studies employing three-way coimmunoprecipitation with Runx2, p62, and LC3 revealed that p62 binds to both LC3 and Runx2 upon metformin treatment in VSMCs. Furthermore, immunoblotting with LC3 revealed that Runx2 specifically binds with p62 and LC3-II in metformin-treated calcified VSMCs. Lastly, we investigated the importance of the autophagy pathway in vascular calcification in a clinical setting. Ex vivo clinical analyses of calcified diabetic lower limb artery tissues highlighted a negative association between Runx2 and LC3 in the vascular calcification process. These studies suggest that exploitation of metformin and its analogues may represent a novel therapeutic strategy for clinical intervention through the induction of AMPK/Autophagy Related 3 (Atg3)-dependent autophagy and the subsequent p62-mediated autophagic degradation of Runx2.

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KEYWORDS

autophagy, calcification, metformin, Runx2, VSMCs

1 | INTRODUCTION

Vascular calcification is a life-threatening complication of cardiovascular disease, affecting tissues including arteries, heart valves and cardiac muscle (Dweck et al., 2012; Margolis et al., 1980; Schmermund et al., 2000; Wayhs et al., 2002). Arterial calcification is recognized as an active, tightly regulated process, sharing many similarities with physiological bone formation (Zhu et al., 2012) and involves the deposition of hydroxyapatite crystals in arteries. Indeed vascular smooth muscle cells (VSMCs), the predominant cell type involved in vascular calcification, can undergo transdifferentiation to a chondrocytic, osteoblastic, and osteocytic phenotype in a calcified environment (Shroff & Shanahan, 2007; Zhu et al., 2011). Furthermore, it has been demonstrated that phosphate (Pi) accelerates this phenotypic trans-differentiation, evident in the loss of characteristic smooth muscle markers, and the development of osteogenic factors, including the master transcription factor runt-related transcription factor 2 (Runx2), and its downstream osteogenic targets osterix, osteocalcin, and bone sialoprotein (Javed et al., 1999; Tyson et al., 2003). Runx2 is crucial for osteogenesis in the skeleton (Takarada et al., 2013); however, it is also expressed in a variety of soft tissues (Jeong et al., 2008). In the vascular system, Runx2 is upregulated at sites of calcification, and targeted ablation of Runx2 decreases expression of its osteogenic targets and reduces calcification (Lin et al., 2015, 2016; Sun et al., 2012).

The biguanide metformin has been used in type 2 diabetes treatment for more than 60 years, and is currently the most common treatment for T2D worldwide (Rena & Lang, 2018). Its therapeutic effects are primarily derived from increasing hepatic adenosine monophosphate-activated protein kinase (AMPK) activity, decreasing gluconeogenesis and lipogenesis (Ghosh et al., 2015). However, there is growing evidence that the beneficial health benefits of metformin extend beyond its capacity to modulate glucose metabolism (Campbell et al., 2017). Metformin has been shown to decrease the incidence of cardiovascular disease in T2D patients (Johnson et al., 2005), reduce atherosclerosis in prediabetic patients (Sardu et al., 2019) and is associated with a lower below-the knee arterial calcification score (Mary et al., 2017), suggesting an important protective effect. Recent studies have also postulated a role for metformin in preventing or regressing abdominal aortic aneurysm formation (Golledge et al., 2019). It has also been shown to improve vascular endothelial function (Nafisa et al., 2018) and inhibit cardiac remodeling (Chen et al., 2018; Sasaki et al., 2009). These studies suggest that metformin has a cardiovascular protective effect, however a comprehensive understanding of the mechanism of action is still lacking.

Studies have recently revealed that metformin exerts direct beneficial effects on VSMC function through the regulation of vascular calcification. Administration of metformin markedly decreases atherosclerotic calcification and Runx2 expression in *ApoE*^{-/-} mice, however,

this protective action is attenuated in *ApoE*^{-/-}/*AMPKα1*^{-/-} mice (Cai et al., 2016). In vitro studies in rat aortic smooth muscle cells have further indicated that metformin inhibits vascular calcification through the AMPK/endothelial nitric oxide synthase/nitric oxide-signaling pathway (Cao et al., 2013). Additionally, metformin has been shown to alleviate VSMC calcification via AMPK-activated autophagy, with an associated decrease in Runx2 expression (Qiu et al., 2021), however, the specific pathways that link these observations remain elusive.

In the present study, we hypothesized that metformin protects against arterial calcification through the direct autophagic degradation of Runx2. We employed clinical analyses in conjunction with in vitro models of arterial calcification to show for the first time that metformin exerts protective effects against vascular calcification through the induction of AMPK/Autophagy Related 3 (Atg3)-dependent autophagy and the subsequent p62-mediated autophagic degradation of Runx2.

2 | MATERIALS AND METHODS

2.1 | Human tissue

Arterial samples were harvested from patients undergoing lower limb amputation for the complications of peripheral artery disease. Past medical history for all patients included type 2 diabetes mellitus, in addition to other cardiovascular risk factors (smoking, hypertension, hyperlipidaemia). Immediately after amputation of the limb in theater, 2 cm length sections of the crural arteries (anterior tibial, posterior tibial, and peroneal) were obtained from the discarded specimen, and placed into paraformaldehyde (PFA) for subsequent processing. Details (age, sex, and comorbidity status) of the patients are provided in Supporting Information: Table 1.

2.2 | VSMC isolation, culture, and calcification

Primary aortic VSMCs were isolated from 5-week-old mice as described previously (Zhu et al., 2016). Mice were euthanized by cervical dislocation. The aorta was then dissected, the adventitia removed, and the aorta cut open to expose the endothelial layer. Eight aortas were pooled together and incubated for 10 min at 37°C in 1 mg/ml trypsin (Thermo Fisher Scientific) to remove any remaining endothelial cells. Aortas were then incubated overnight at 37°C in VSMC growth medium containing α -MEM (Life Technologies), 10% fetal bovine serum, and 1% gentamycin (Thermo Fisher Scientific). Tissues were then digested with 425 U/ml collagenase type II (Worthington Biochemical Corporation) for 4 h at 37°C. The resulting cell suspension was centrifuged at 2000g for 5 min. VSMC pellets were resuspended in culture medium and cultured for two passages in T25 tissue culture flasks coated with 10 μ g/ml laminin (Sigma) to promote maintenance of the contractile differentiation state.

VSMCs were seeded in growth medium at a density of $3\text{--}5 \times 10^4$ cells per well in 12-well plates (Corning Inc.). Calcification was induced as described previously (Zhu et al., 2016). In brief, cells were grown to confluence (Day 0) and changed to calcification medium, which was prepared by supplementing growth medium with inorganic phosphate (Pi) to a final concentration of 3 mM. Cells were cultured in calcifying media for up to 7 days, and the medium changed every second/third day. For all the experiments, $N = 3$ is representative of 3 independent experiments pooled from 8 aortas.

2.3 | Determination of calcification

Calcium deposition was quantified by HCl leaching, as previously described (Zhu et al., 2016). Briefly, cells were washed with phosphate buffered saline (PBS) and incubated for 24 h in 0.6 N HCl at 4°C. Calcium content was determined calorimetrically by a stable interaction with O-Cresolphthalein using a commercially available kit (Randox Laboratories Ltd) and corrected for total protein concentration (Bio-Rad Laboratories Ltd). Calcium deposition was also evaluated by alizarin red staining (Roberts et al., 2021). Cells were washed twice with PBS, fixed in 10% neutral buffered formalin (NBF) for 15 min, stained with 2% alizarin red (pH 4.2) for 5 min at room temperature, and rinsed with distilled water.

2.4 | Transfection assays

VSMCs were transfected with 60 pmol of mouse *Atg3* (Santa Cruz Biotechnology) or scrambled control siRNA (Santa Cruz Biotechnology) with siRNA transfection reagent (Santa Cruz Biotechnology), according to the manufacturer's instructions. The cells were harvested for experiments up to 48 h posttransfection and subsequently cultured in calcification medium in the presence of metformin for 72 h.

2.5 | Cell imaging

Cells were seeded on glass cover slips in 24-well plates at a density of 3×10^6 cells/well. Cells were fixed with 4% PFA at 4°C and washed with PBS. The fixed cells were permeabilised with 0.1% triton X100 (Sigma) and blocked with 2% goat before incubating with primary antibodies LC3 (1:300; rabbit polyclonal; PM036; MBL International) or Runx2 (1:100; Mouse; Sc-390351; Santa Cruz Biotechnology) overnight at 4°C. After washing cells were incubated for 1 h in the dark with Alexa Fluor@488 anti-rabbit antibody (A11034; Life Technologies) and Alexa Fluor@647 goat anti mouse antibody (A21236; Life Technologies). Cells were then washed with PBS and stained with Hoechst (1:10,000; Sigma). Glass coverslips were mounted onto slides with Prolong® Gold Anti-Fade Reagent containing DAPI (Life Technologies). Fluorescence signal was detected under a Zeiss LSM 710 inverted confocal microscope. ImageJ was used to determine the number of LC3 puncta in the cytoplasm and Runx2 intensity in the nucleus.

2.6 | Immunofluorescence for tissue sections

Tissues were fixed in 10% NBF for 24 h before being dehydrated, embedded in paraffin wax, and sectioned (4 µm) using standard procedures as previously described (Zhu et al., 2016). Sections were dewaxed in xylene and stained with Von Kossa and alizarin red (Sigma) to visualize phosphate and calcium deposition, respectively. For immunofluorescence analysis, sections were demasked with 10 mM sodium citrate buffer. Endogenous peroxidase and non-specific antibody binding were blocked before overnight incubation with primary antibodies LC3 (1:300; PM036; MBL International), Runx2 (1:200; Sc-390351; Santa Cruz Biotechnology), or ATG3 (1:300; ab108251; Abcam) at 4°C. The sections were then incubated for 1 h in the dark with Alexa Fluor@488 anti-rabbit antibody (Life Technologies; A11034) and Alexa Fluor@647 goat anti mouse antibody (Life Technologies; A21236). Sections were washed in PBS and stained with Hoescht (1:10,000; Sigma) and then mounted onto slides with Prolong® Gold Anti-Fade Reagent (Life Technologies). Fluorescence signal was detected under a Zeiss LSM 710 inverted confocal microscope. Control sections were incubated with non-immune goat IgG (2 µg IgG/ml) in place of the primary antibody.

2.7 | Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer supplemented with Protease Inhibitor Cocktail (Thermo Fisher Scientific) and total protein concentration determined (Thermo Fisher Scientific). Immunoblotting was performed as previously described (Zhu et al., 2016). Nitrocellulose membranes were probed overnight at 4°C with primary antibodies (1:1000 dilution in LICOR blocking buffer or 5% milk in TBST) LC3 (PM036; MBL International), *Atg3* (ab108251; Abcam), Runx2 (ab236639; Abcam), AMPKα (D5A2; Cell Signaling Technology), Phospho-AMPKα (Thr172) (40H9; Cell Signaling Technology), osterix (AF7580; Affinity Biosciences), Osteocalcin (ab93876; Abcam), bone sialoprotein (DF7738; Affinity Biosciences). Blots were subsequently incubated in goat anti-rabbit IRDye 680RD (926-68071; Thermo Fisher Scientific) or HRP conjugated goat anti-rabbit IgG (P0449; Dako) for 1 h. Blots were then imaged using an Odyssey CLx Infrared Imaging System (Li-COR) or developed by the GeneGenome system (Syngene). Membranes were then washed reprobed for β-actin expression (1:1000; 4970; Cell Signaling Technology). For *Atg3* studies, β-actin expression was determined on a parallel membrane due to molecular weights.

2.8 | Coimmunoprecipitation (co-IP) studies

Cells were lysed in Lysis buffer (Cell Signaling Technology) supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific) and total protein concentration determined (Thermo Fisher Scientific). Cell lysates (500 µg) were incubated for 12 h at 4°C either with 5 µg/ml anti-Runx2 (ab236639; Abcam), 5 µg/ml anti-p62 (ab240635; Abcam), or 5 µg/ml anti-LC3 (PM036; MBL international)

or 5 µg/ml anti-rabbit IgG (7074; Cell Signaling Technology). Subsequently the lysates were incubated with 20 µl Protein G magnetic agarose beads (73778; Cell Signaling Technology) for 30 min at room temperature. Protein bound to the beads was washed five times with lysis buffer, pelleted using a magnetic rack and boiled for 8 min in NuPAGE LDS sample buffer with NuPAGE sample reducing agent (Thermo Fisher Scientific) before analysis by immunoblotting with Runx2 (ab236639; Abcam), p62 (ab240635; Abcam), and LC3 (PM036; MBL international) antibodies as described above.

2.9 | Gold immunolabeling and transmission electron microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), then blocked with 5% goat serum (Sigma) for 1 h before incubating with anti-Runx2 antibody (1:25; Abcam ab236639) for 1 h, followed by 10 nm Colloidal Gold Conjugated Goat Anti-mouse IgG (H + L) secondary antibody (1:400; Sigma) for 1 h. Samples were viewed on a JEOL 1400/JEM plus (JEOL) with AMT UltraVUE camera (AMT) and Gatan OneView camera (Gatan).

2.10 | Statistical analysis

All data are presented as mean ± SEM. Data were analyzed by unpaired *t*-test or one-way analysis of variance followed by Tukey's range test, as appropriate. All statistical analysis was performed using GraphPad prism software. $p < 0.05$ were considered to be significant, and p values are represented as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3 | RESULTS

3.1 | Metformin protects against vascular calcification through autophagic degradation of Runx2

We initially examined the effects of metformin on the calcification of VSMCs. Since arterial calcification is highly correlated with elevated serum Pi levels, VSMCs were cultured in growth medium containing high (3 mM) Pi as previously described (Zhu et al., 2016). Calcification was blunted in VSMCs by metformin in a dose-dependent manner (0.5–1.5 mM) compared to control cells (Figure 1a,b; $p < 0.01$). 1 mM metformin was selected for subsequent studies. Next, we investigated if metformin is able to reduce the expression of the osteogenic markers osterix, osteocalcin, and bone sialoprotein under elevated Pi treatment. VSMCs cultured under high Pi conditions in the presence of metformin (1 mM) showed a significant reduction in osterix, osteocalcin, and bone sialoprotein protein expression compared to control cells (Figure 1c,d; $p < 0.05$). We further investigated if

metformin was able to activate AMPK under elevated Pi treatment. VSMCs cultured under high Pi conditions in the presence of metformin (1 mM) showed a significant increase in p-AMPK compared to control cells (Figure 1e,f; $p < 0.05$).

We subsequently assessed whether metformin treatment reduces calcium deposition in VSMCs through altered autophagic flux. VSMCs cultured under high Pi conditions were treated with bafilomycin-A1 (Baf-A; 5 nM). Combined metformin and Baf-A treatment blocked lysosomal function, leading to the build-up of LC3-II (punctate LC3) within autophagosomes and autolysosomes in the cytoplasm (Figure 2a).

VSMCs cultured under high-phosphate conditions in the presence of metformin (1 mM) showed a significant increase in LC3 puncta following Baf-A treatment compared to control cells (Figure 2a,b; $p < 0.001$). Furthermore, reduced expression of Runx2 was observed in the nuclei of metformin-treated calcifying VSMCs (Figure 2a,c; $p < 0.0001$), suggesting a negative association between LC3 and Runx2 expression in the nucleus. However, Runx2 expression was noted in the cytoplasm surrounded by punctate LC3 in the VSMCs treated with metformin and Baf-A in combination (Figure 2a,d; $p < 0.0001$). Furthermore, the presence of Runx2 inside the double-membrane autophagosomes/autolysosomes following metformin (1 mM) treatment was further confirmed by transmission electron microscopy (Figure 2e,f). We also assessed the levels of p62 (SQSTM-1) a marker of autophagic degradation. A reduction in p62 expression was observed in VSMCs treated with metformin, suggesting increased autophagic degradation with metformin treatment (Figure 2g,h; $p < 0.01$). Together these data suggest that metformin exerts its protective effects against VSMC calcification by enhancing autophagic flux, and the subsequent transit of Runx2 via autophagosomes/autolysosomes for autophagic degradation.

3.2 | Silencing Atg3 leads to increased accumulation of Runx2 in the nucleus of metformin-treated calcifying VSMCs

We next evaluated the functional role of autophagy in the regulation of Runx2 during vascular calcification through autophagy-related 3 (Atg3) knockdown in murine VSMCs. Atg3 is an E2 ubiquitin-conjugating enzyme which catalyses the conjugation of LC3-I with phosphatidylethanolamine to form LC3-II, a key step in autophagosome formation (Yamada et al., 2007).

Atg3 knockdown in VSMCs (Figure 3a,b, $p < 0.05$) failed to induce autophagy (punctate LC3) following metformin treatment (1 mM; Figure 3c,d, $p < 0.001$). Atg3 knockdown in VSMCs also aggravated Pi-induced calcification (Figure 3e, $p < 0.0001$). Furthermore, Atg3 knockdown induced a significant increase in Runx2 expression in VSMCs cultured under high Pi conditions in the presence of metformin in the nucleus compared to Scr control cells (Figure 3f,g; $p < 0.0001$). Together, these data suggest that nuclear expression of Runx2 is regulated by autophagy following metformin treatment of calcifying VSMCs.

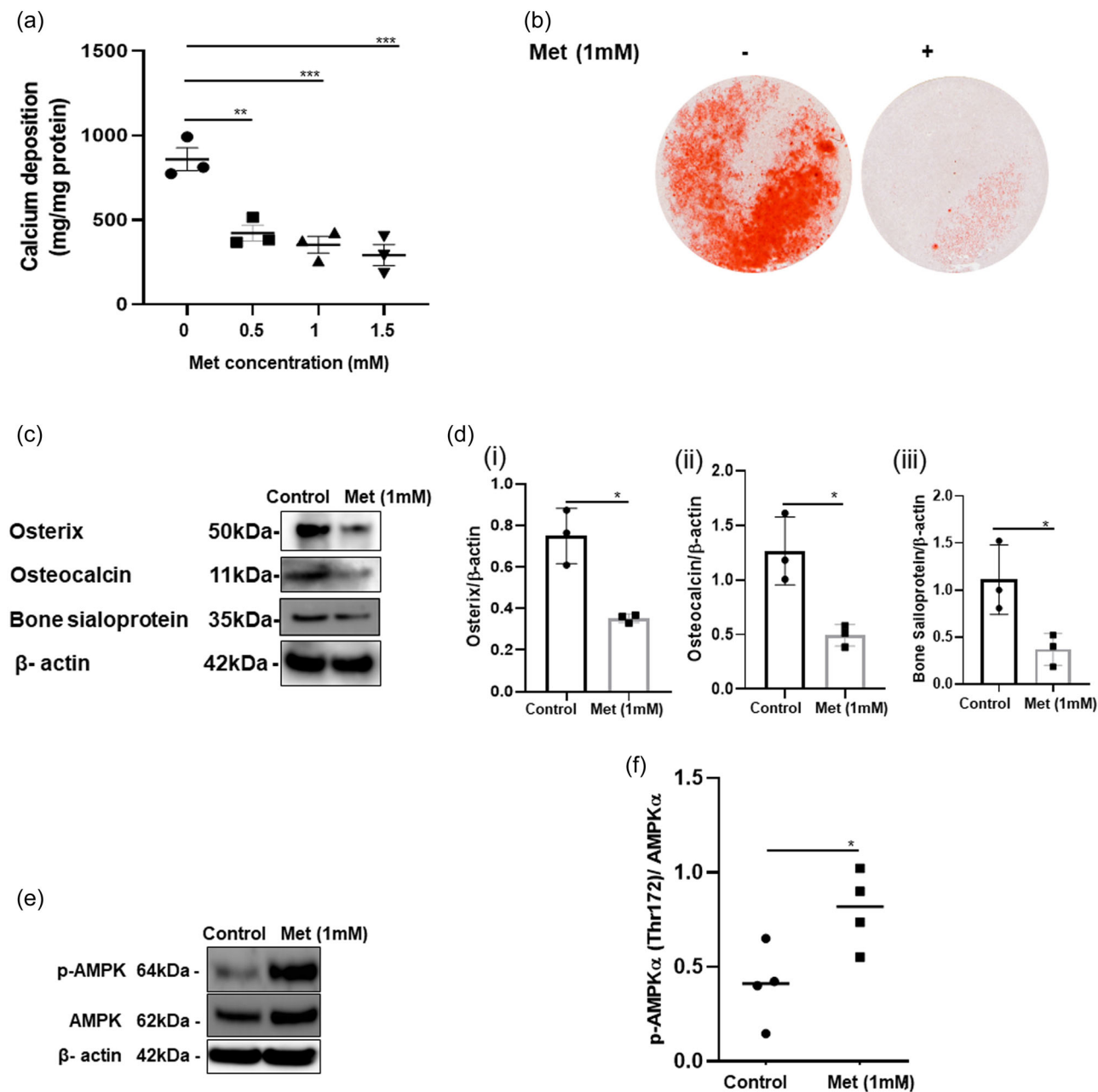


FIGURE 1 Metformin activates AMPK and calcification in VSMCs. VSMCs were cultured with high phosphate (3 mM Pi) and metformin (met) for 7 days. (a) Calcium content (mg/mg protein) of cells treated with metformin (0.5–1.5 mM), (n = 3). (b) Representative alizarin red images of cells treated with metformin (1 mM). (c) Representative immunoblots (d) immunoblot quantification showing the effect of metformin (1 mM) on expression of osteogenic markers osterix, osteocalcin, and bone sialoprotein compared with β -actin (n = 3). (e) Representative immunoblots and (f) immunoblot quantification showing the effect of metformin (1 mM) on the phosphorylation of AMPK compared with total AMPK (n = 4). AMPK, adenosine monophosphate-activated protein kinase; VSMCs, vascular smooth muscle cells.

3.3 | Metformin induces p62 mediated sequestering of Runx2 in the autophagosomes

We next sought to elucidate the mechanism through which the autophagy pathway inhibits vascular calcification following metformin treatment. We hypothesized that on AMPK activation Runx2 is sequestered by autophagosomes via the classical receptor of autophagy p62 (also known as SQSTM-1). p62

selectively recognizes autophagic cargo and mediates their engulfment into autophagosomes by binding small ubiquitin-like modifiers (Rogov et al., 2014).

Protein lysates were analyzed from VSMCs cultured with Baf-A (5 nM) under control and calcified conditions, in the presence or absence of metformin (1 mM). Three-way co-IP with Runx2, p62, and LC3 revealed that p62 binds to both LC3 and Runx2 upon metformin treatment in VSMCs (Figure 4a–c). Furthermore,

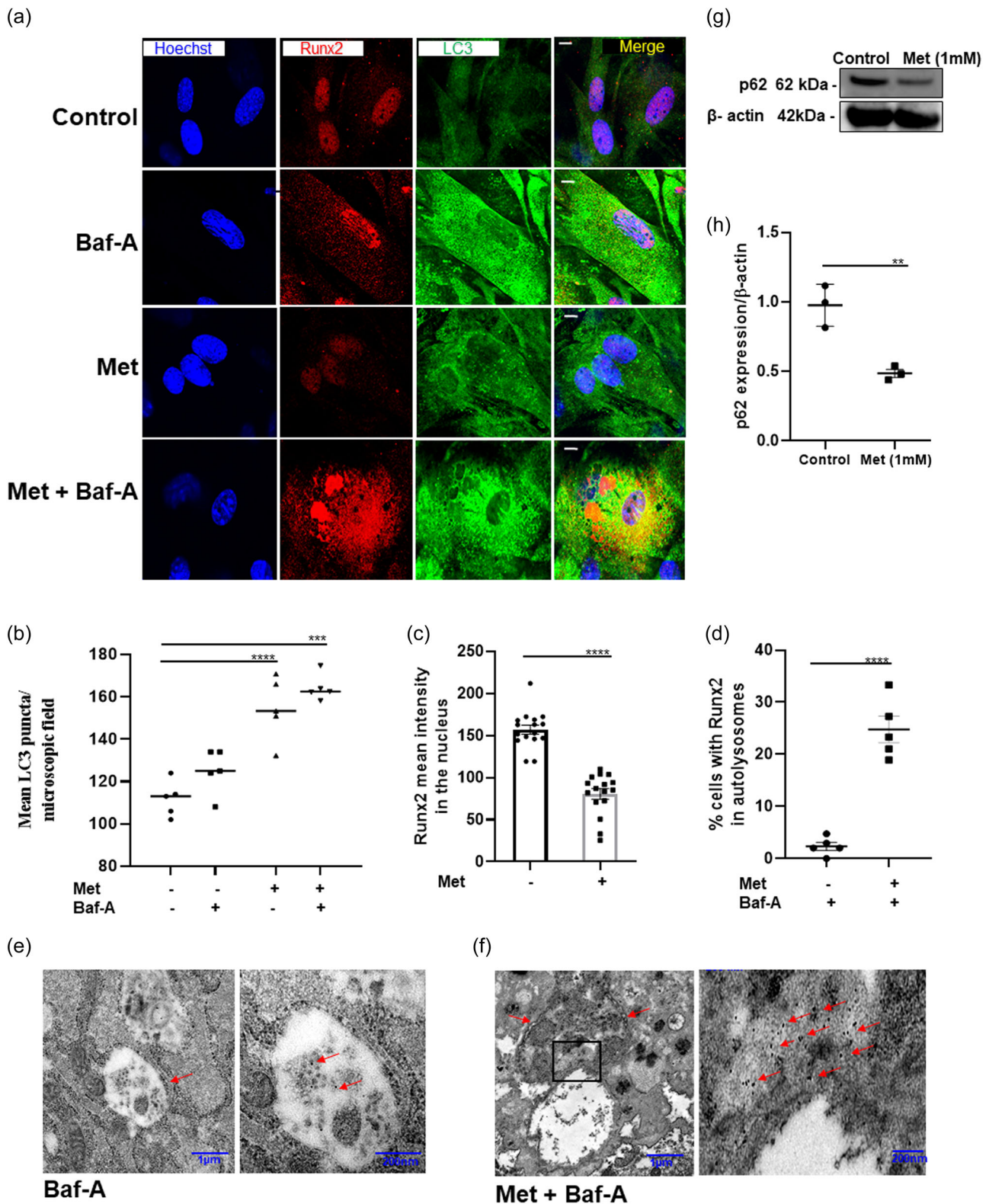


FIGURE 2 Metformin protects against vascular calcification through autophagic degradation of RUNX2. VSMCs were cultured with high phosphate (3 mM Pi) and met (1 mM) for 7 days. (a) Representative confocal images showing the effect of bafilomycin (Baf-A; 5 nM) and/or met (1 mM) treatment on Runx2 and LC3 expression ($n = 3$; scale bar = 20 μm) with quantification of (b) LC3 puncta (c) nuclear Runx2 and (d) percentage (%) cells with Runx2 in autolysosomes. Representative transmission electron microscopy images showing (e) reduced Runx2 in control cells versus, zoomed in to show the Runx2 gold labeling (f) double membraned autophagosomes (blue arrows) and zoomed in square box are with Runx2 gold labeling (red arrows) in the autophagosomes with metformin (1 mM) treatment. (g) Representative immunoblots and (h) immunoblot quantification showing the effect of metformin (1 mM) on p62 expression compared with β -actin ($n = 3$). Runx2, runt-related transcription factor 2; VSMCs, vascular smooth muscle cells.

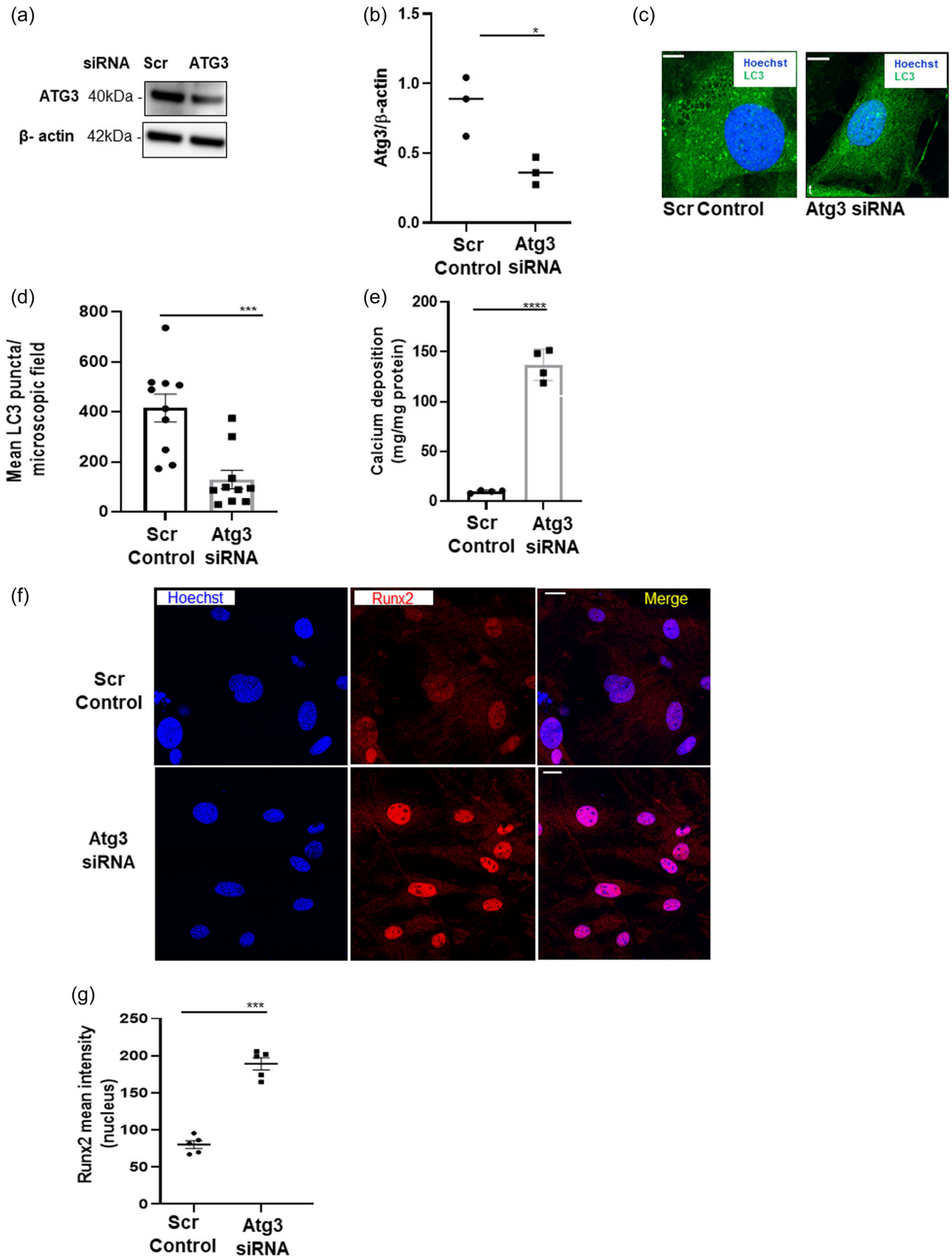


FIGURE 3 (See caption on next page)

immunoblotting with LC3 revealed that Runx2 specifically binds with p62 and LC3-II (presence of 15 kDa band only) in metformin-treated calcified VSMCs, with binding absent in control cells (Figure 4c). We further investigated if metformin enhances the expression of K63, as K63 ubiquitination enhances sequestration of autophagic cargo by linking to p62 (Wurzer et al., 2015). Enhanced expression of K63 was observed in calcified VSMCs treated with metformin (Figure 4d). Together these data strongly indicate that metformin enhances autophagic flux and selectively engulfs Runx2 for degradation in active autophagosomes marked with LC3-II via the p62 autophagy receptor (Figure 4e).

3.4 | Reduced expression of ATG3 and LC3 in calcified human vascular tissue

To investigate the importance of the autophagy pathway in vascular calcification in a clinical setting, localization studies were undertaken. Calcification of lower limb artery tissue was confirmed by alizarin red and Von Kossa staining (Figure 5a-d). In addition, calcified artery tissues showed reduced expression of the autophagy markers ATG3 (Figure 5f) and LC3 (Figure 5h) compared to non-calcified control samples (Figure 5e and 5g, respectively). Together these results indicate a reduction in autophagic flux in human calcified cardiovascular tissue.

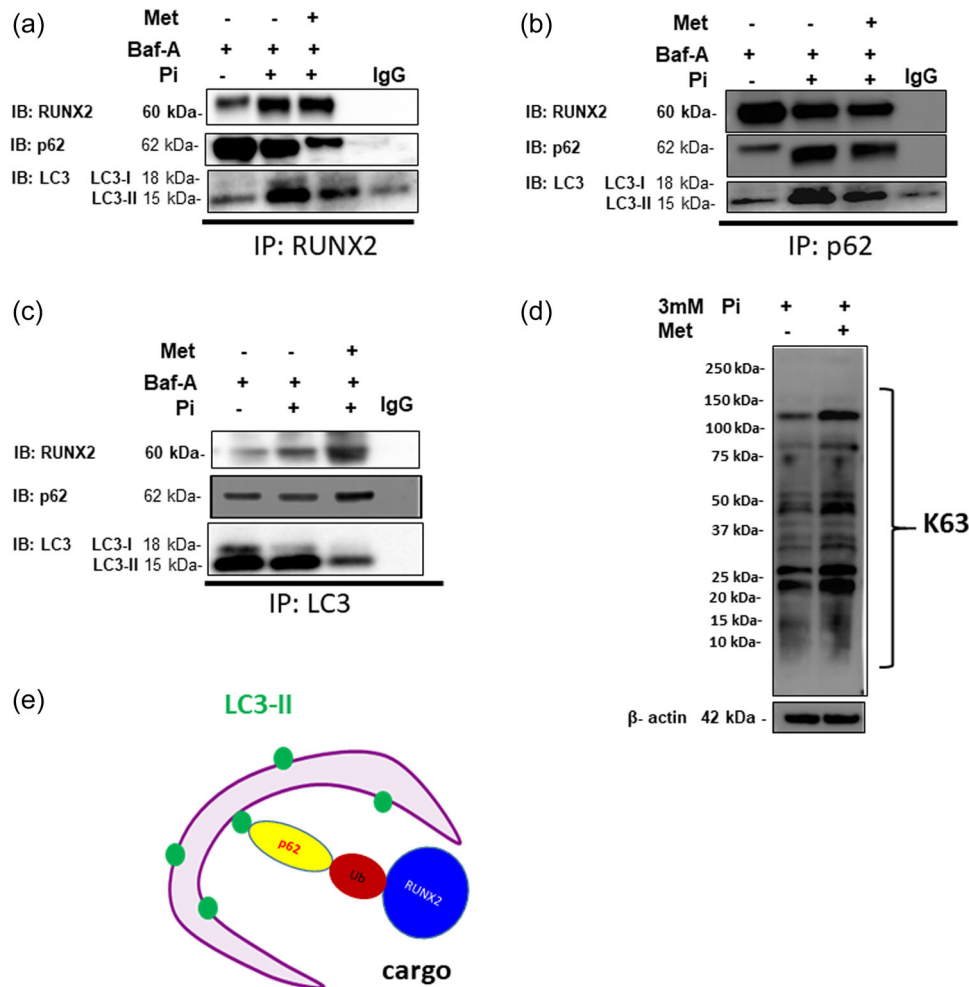
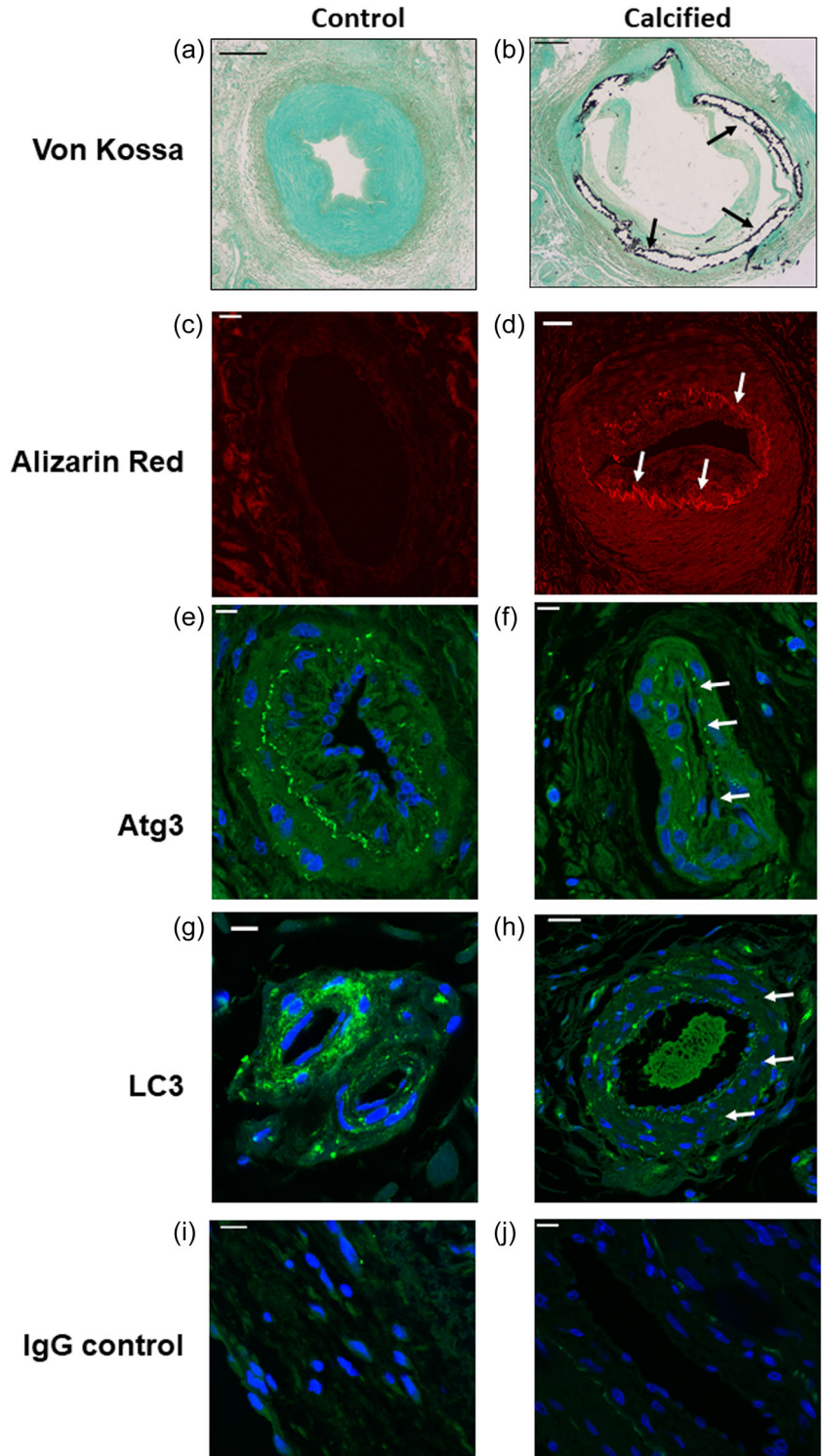


FIGURE 4 Metformin induces p62 mediated sequestering of Runx2 in VSMC autophagosomes. VSMCs were cultured in growth medium or treated for 7 day with 3 mM phosphate (Pi), 1 mM met and/or 5 nM bafilomycin (Baf-A). (b) Representative immunoblots for Runx2, p62, and LC3 with (a) Runx2 co-IP lysate (b) p62 co-IP lysate (c) LC3 co-IP lysate and (d) K63 expression. Rabbit IgG was used as a negative control ($n = 2$). (e) Proposed LC3-p62 interaction with Runx2 inside an autophagosome. co-IP, coimmunoprecipitation; Runx2, runt-related transcription factor 2; VSMCs, vascular smooth muscle cells.

FIGURE 3 Atg3 knockdown leads to increased accumulation of Runx2 in the nucleus of VSMCs cultured with metformin. VSMCs were transfected with either scrambled (siScr) or Atg3 (siAtg3) siRNA. (a) After 96 h, cellular Atg3 content was determined by immunoblotting (b) quantification of reduction in Atg3 protein compared with scrambled siRNA control ($n = 3$). The effect of siScr and siAtg3 treatment in the presence of met (1 mM) and 3 mM Pi on (c) LC3 expression (scale bar = 20 μ m) with quantification of (d) LC3 puncta ($n = 3$), (e) calcium deposition ($n = 4$) (f) Runx2 expression with Hoechst staining of DNA shown by representative confocal images with (g) quantification of the nuclear staining intensity of Runx2 ($n = 3$). Runx2, runt-related transcription factor 2; VSMCs, vascular smooth muscle cells.

FIGURE 5 Reduced expression of ATG3 and LC3 in calcified lower limb artery tissue from type 2 diabetes mellitus patients. Calcification of human lower limb artery tissue was confirmed by (a, b) Von Kossa (arrows indicate positive staining) and (c, d) alizarin red (arrows indicate positive staining). (e, f) Atg3 and (g, h) LC3 expression (arrows) was reduced in calcified compared to control tissues. (i, j) Rabbit IgG control. $n = 3$, scale bar = 10 μ m.



3.5 | Loss of interaction between Runx2 and LC3 in CAVD and diabetic arterial calcification

Finally, to assess whether the targeting of Runx2 by metformin could be a viable therapeutic strategy, we next performed further clinical analyses to ascertain whether Runx2 and LC3 are central to the etiology of diabetic arterial calcification. Immunohistochemical staining revealed increased expression of RUNX2 in lower limb

artery tissue. In the control tissues, increased expression of LC3 was seen with low levels of Runx2 expression (Figure 6d). However, almost no LC3 puncta were seen in calcified artery tissues with high Runx2 expression (Figure 6h).

In summary, our *in vitro* investigations establish for the first time that metformin exerts protective effects against vascular calcification through the induction of autophagy and the subsequent restoration of the interaction between Runx2 and LC3 (Figure 7). Our subsequent ex

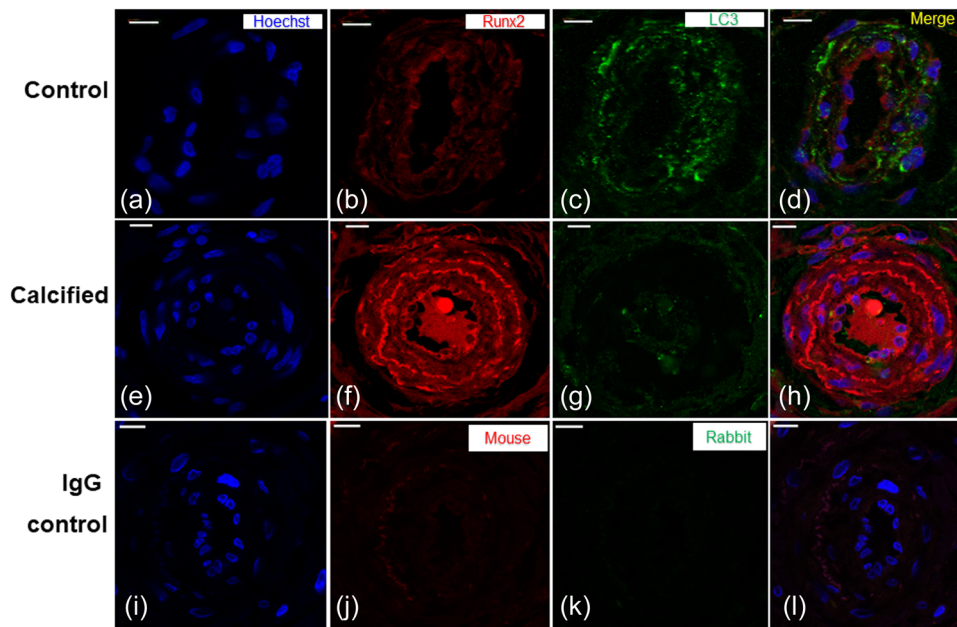


FIGURE 6 Negative association between Runx2 and LC3-II in calcified lower limb artery tissue from type 2 diabetes mellitus patients. (a, e) Hoechst staining of DNA (b, f) Runx2 expression was increased whereas (c, g) LC3 expression was reduced in calcified compared to control tissue (d, h) merged images, (i–l) mouse and rabbit IgG control. $n = 3$, scale bar = 10 μm . Runx2, runt-related transcription factor 2.

vivo clinical analyses highlight a negative association between Runx2 and LC3 in the vascular calcification process and suggest that exploitation of metformin and its analogues may represent a novel therapeutic strategy for clinical intervention.

4 | DISCUSSION

It is well established that metformin, the most common treatment for type 2 diabetes, mediates changes in vascular function, structure and growth (Deng et al., 2020). Furthermore, studies in VSMCs have reported a beneficial role of metformin in atherosclerosis by inhibiting the proliferation, calcification, and inflammation of VSMCs (Deng et al., 2020). Whilst the mechanisms underpinning these novel actions of metformin have yet to be fully elucidated, recent findings suggest that metformin may exert its cardio-protective effects via increased autophagic activity (Qiu et al., 2021; Xie et al., 2011). In the present study, we have employed an in vitro model of arterial calcification to show for the first time that metformin alleviates calcification through induction of p62-mediated sequestering of the osteogenic transcription factor Runx2 in autophagosomes.

Autophagy is a multifunctional process involved in numerous cellular activities (Mizushima et al., 2008) and is essential for cellular development, differentiation, and survival (Levine & Klionsky, 2004). Indeed, autophagy has been shown to play an important role in not only the physiological function of VSMCs but also the etiology of cardiovascular disease (Deng et al., 2020). Recently autophagy has been identified as a novel adaptive mechanism that protects against

VSMC calcification by regulating apoptosis and the release of calcifying matrix vesicles from VSMCs (Dai et al., 2013; Phadwal et al., 2020). The present study offers further insight into the role of autophagy in vascular calcification. We confirm and extend data generated by Qiu et al. (2021) in the rat A7r5 thoracic aorta VSMC cell line, by employing a more physiologically relevant primary cell culture model. We demonstrate that metformin alleviates the calcification of murine aortic VSMCs by promoting autophagic activity, as indicated by an increased number of autophagosomes, green fluorescent LC3 puncta and LC3II/I expression in metformin-treated VSMCs compared to control cells.

A number of autophagy-related (Atg) proteins, which are indispensable for autophagosome formation, have been previously shown to be associated with vascular calcification including Atg4 (b), Atg5; Atg7, Atg12, and Atg16 (Peng et al., 2017; Zhou et al., 2021). In the present study, we reveal for the first time a functional contribution for Atg3 in the vascular calcification process. Autophagy inhibition by siRNA knockdown of Atg3 notably aggravated Pi-induced calcium deposition in VSMCs. Furthermore, Atg3 knockdown markedly blunted the anti-calcification effects of metformin. Atg3 is one of the key upstream molecules required for autophagy, and its homologs are common in eukaryotes (Agrotis et al., 2019; Sou et al., 2008). Additionally, Atg3^{-/-} mice are nonviable, suggesting that Atg3 is essential for the homeostasis of the organism (Sou et al., 2008). Atg3 contributes to autophagosome formation by interacting with Atg7, Atg8, Atg12, and the lipid membrane (Fang et al., 2021). Atg3 also contributes to phagophore elongation, acting as an E2 ubiquitin-like conjugating enzyme in the Atg8 conjugation system (Hervas et al., 2017).

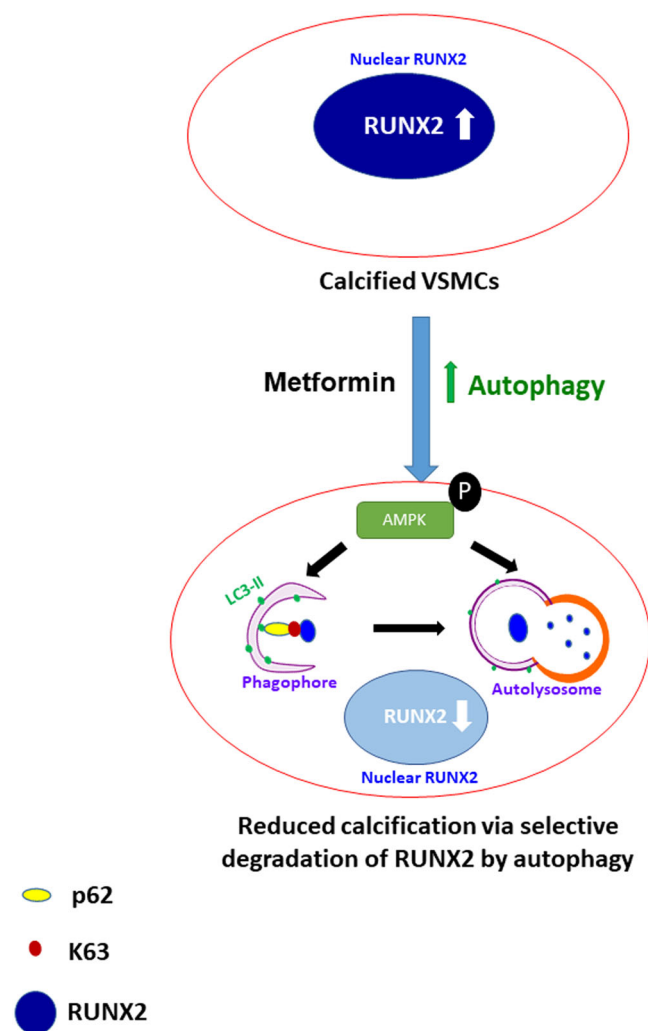


FIGURE 7 Schematic representation showing the proposed mechanism through which metformin reduces vascular calcification by selective degradation of Runx2 by autophagy. Runx2, runt-related transcription factor 2.

In the present study we demonstrate that metformin can activate AMPK, a critical cellular energy sensor, in primary murine VSMCs cultured under calcifying conditions, confirming published reports in osteoblasts (Kanazawa et al., 2018) and the A7r5 VSMC cell line (Qiu et al., 2021). Our data also support the recent demonstration that AMPK initiates autophagy indirectly by deactivating mTORC1 following metformin treatment of calcifying A7r5 cells and contribute to autophagosome maturation and their fusion with lysosomes (Jang et al., 2018) (Figure 7). Together, these data support a growing body of evidence highlighting an essential role for AMPK in the vascular calcification process, through multiple mechanisms including Runx2 signaling (Cao et al., 2013), triggering autophagy (Kanamori et al., 2019; Xu et al., 2021), attenuating endoplasmic reticulum stress (Li et al., 2019), and activating endothelial nitric oxide synthase (Cao et al., 2013; Eriksson & Nystrom, 2014).

Our *in vitro* investigations further revealed that metformin treatment reduces the expression of Runx2, a recognized regulator of

VSMC osteogenic transition and the expression of its downstream targets osteonin, osteocalcin, and bone sialoprotein. Indeed, there is a substantial body of evidence linking Runx2 upregulation with vascular calcification *in vitro* (Takarada et al., 2013), whilst studies utilizing VSMC-specific Runx2 deletion using SM22-recombinase transgenic allele mice have showed that Runx2 expression is required in VSMCs for arterial calcification *in vivo* (Lin et al., 2016). Specifically, the nuclear localization of Runx2 is associated with the early transformation into osteoblast-like cells (Sikura et al., 2019). Metformin has been previously shown to alleviate VSMC calcification via autophagy, with a simultaneous decrease in Runx2 expression (Qiu et al., 2021); however, the specific pathways underpinning these observations have yet to be elucidated. In the present study, we have employed defined co-IP studies to reveal for the first time that metformin directly attenuates Runx2 action in VSMCs via p62, an autophagosome cargo protein that targets other proteins that bind to it for selective autophagy. These data progress previous work reporting an association between autophagy and p62 in VSMC calcification (Ma et al., 2019). Our *in vitro* findings are further supported by clinical analyses, which reveal reduced autophagic flux and a negative correlation between the expression of LC3 and Runx2 in the diabetic calcified artery tissues.

The metformin dosage used in the current study (1 mM) and in additional *in vitro* experiments (0.5 mM) (Cao et al., 2013; Qiu et al., 2021) can be correlated to the high dosage of metformin (>1700 mg/day) used in human clinical trials. Interestingly, only this high dosage was able to reduce triglyceride levels and high-density lipoprotein function, which may contribute to the anti-atherosclerotic effect (Luo et al., 2019). Furthermore, the beneficial effects of metformin are not limited to T2D patients alone. Recent clinical trials have shown that metformin can reduce myocardial ischemia in female patients with angina (Jadhav et al., 2006) and carotid intima-media thickness in nondiabetic patients (Meaney et al., 2008). Together with atorvastatin, metformin can also improve the rate of obesity and subclinical inflammation (Maruthur et al., 2016).

In conclusion, we have undertaken clinical analyses in conjunction with *in vitro* studies to provide fundamental insights into the role of metformin as a potent inhibitor of vascular calcification. Our study suggests that metformin protects against vascular calcification through the autophagic degradation of Runx2. This data may have important health ramifications for diabetic patients receiving metformin, particularly since vascular calcification is a common pathological phenomenon in diabetes (Zhu et al., 2012). The previously established cardiovascular benefits of metformin administration (Deng et al., 2020), in conjunction with the findings from our laboratory and others together may pave the way for preclinical and clinical trials for the treatment of vascular calcification with metformin therapy. We further propose that the mechanism of Runx2 degradation through the p62 adaptor via metformin may also be valid outwith diabetic patients and beneficial in the healthy aging population, as the incidence of vascular calcification increases with aging (Giallauria et al., 2013) whereas autophagic degradation declines with age (Kaushik et al., 2021).

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ETHICS STATEMENT

Informed consent was obtained from all patients involved in the study. The use of human tissue samples was approved by the NHS Lothian Ethics Committee (REC 2002/1/22; 2002/R/OST/02) and NRS BioResource (15/ES/0094, SR719, SR769; 15/SS/0182, SR589).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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