Pathological cartilage calcification plays an important role in osteoarthritis progression but in which the origin of calcified extracellular vesicles (EVs) and their effects remain unknown. Here, we demonstrate that pathological cartilage calcification occurs in the early stage of the osteoarthritis in which the calcified EVs are closely involved. Autophagosomes carrying the minerals are released in EVs, and calcification is induced by those autophagy-regulated calcified EVs. Autophagy-derived microtubule-associated proteins 1A/1B light chain 3B (LC3)–positive EVs are the major population of calcified EVs that initiate pathological calcification. Release of LC3-positive calcified EVs is caused by blockage of the autophagy flux resulted from histone deacetylase 6 (HDAC6)–mediated microtubule destabilization. Inhibition of HDAC6 activity blocks the release of the LC3-positive calcified EVs by chondrocytes and effectively reverses the pathological calcification and degradation of cartilage. The present work discovers that calcified EVs derived from autophagosomes initiate pathological cartilage calcification in osteoarthritis, with potential therapeutic targeting implication.

INTRODUCTION

Osteoarthritis (OA) is one of the leading causes of physical disability. This pathological condition is characterized by progressive degradation of the articular cartilage, remodeling of the subchondral bone, and consequent articular dysfunction (1). To date, there is no effective approach in impeding or reversing the progress of OA. This is because that the pathophysiological mechanism of OA is obscure (2). Calcification is necessary for the formation and function of skeletal tissues. However, in OA and other heterotopic calcification diseases, pathological calcification is observed in the cartilage and soft tissues (3). Recent evidence suggests that abnormal cartilage calcification is an important pathogenic feature that results in the occurrence and development of OA (4). Studies by the authors and other groups showed that cartilage calcification, identified in almost all osteoarthritic joints of patients, strongly correlates with the severity of OA. Cartilage calcification is used for prediction of the morbidity and mortality of patients as the disease progressed (5, 6). Because calcium deposition in the cartilage matrix causes degeneration of articular tissues, inhibition of pathological joint calcification helps arrest OA progression (7, 8). These observations suggest that pathological calcification is a disease initiator instead of being an outcome of OA progression. Nevertheless, the mechanisms underlying the formation of pathological calcification in OA patients remain unknown.

Extracellular vesicles (EVs) are a heterogeneous population of cell-derived, membrane-bound structures that range from ~50 to 1000 nm in size (9). Although initially considered as inert cellular debris, EVs are now recognized as important mediators in intercellular communication. EVs deliver their cargoes through protein-ligand interactions or fusion with the plasma membrane of recipient cells. They have been implicated in the mineralization of calcified cartilage, bone, and dentin and are often associated with crystals of calcium phosphate mineral, which are coined calcified EVs (10). EVs driving calcification in mineralizing tissues were first identified by Anderson (11) and Bonucci (12) in 1967 and have been termed as matrix vesicles. Calcified EVs derived from growth plate chondrocytes nucleate mineral crystals and initiate the mineralization cascade. This results in mineralization of the extracellular matrix (ECM) in endochondral ossification (13). Recently, calcified EVs derived from macrophages and smooth muscle cells (SMCs) have also received increased attention for their role in vascular calcification (14). Those EVs function as nucleating foci for the formation of microcalcifications within atherosclerotic plaques. This results in plaque instability, rupture, and consequently myocardial infarction and stroke (15). Although EV–like particles were ultrastructurally detected in articular cartilage (16), their origin and roles in osteoarthritic cartilage calcification have not been elucidated.

Autophagy is a cytoplasmic autodigestive process that degrades and recycles harmful proteins or injured organelles (17). Our recent studies showed that autophagy plays a major role in transferring calcium phosphate precursors from dysfunctional mitochondria to autophagosomes, and inhibition of autophagy prevents calcification of the ECM by dental pulp stem cells (18). In addition, autophagy has been revealed to act as an unconventional secretion process known as the secretory autophagy (19). Secretory autophagosomes transport proteins outside cells via the EVs in age-related neurodegenerative disease (20), such as the release of α-synuclein in Parkinson’s disease (21). As a marker of autophagosomes, microtubule-associated proteins 1A/1B light chain 3B (LC3) mediates the loading of protein and RNA cargoes into EVs for secretion outside cells in the secretory autophagy pathway (22). However, the relationship between secretory autophagosomes and calcified EVs as well as the manner in which secretory autophagosomes initiate calcification are unknown.
The temporomandibular joint (TMJ) is one of the most frequent joints involved in OA and is biomechanically related to dental occlusion. The authors have developed a TMJ-OA animal model using a unilateral anterior crossbite procedure (23–25). Progressive TMJ degeneration, abnormal remodeling of subchondral bone, and early mineral deposits in the condylar cartilage were observed in this model (26, 27). On the basis of this model and the aforementioned knowledge, the objectives of the present study are to investigate the function of EVs in osteoarthritic cartilage and their contribution in pathological cartilage calcification. The results should provide physicochemical mechanistic insight into the role of autophagosomes-derived calcified EVs in mediating osteoarthritic TMJ cartilage calcification.

RESULTS
Cartilage calcification occurs early in OA and aggravates progressively
Pathological calcification, stained black by Von Kossa (Fig. 1A) or red by Alizarin Red S (Fig. 1B), was seen in the osteoarthritic cartilage as early as the second week. The condition deteriorated at the eighth week (Fig. 1, A and B). Areas occupied by mass-like calcification were significantly increased in both osteoarthritic groups compared with those of the control groups (Fig. 1C). The Osteoarthritis Research Society International (OARSI) histological score in osteoarthritic groups was higher than the age-matched control (fig. S1). When the cartilage was examined by micro–computed tomography (micro-CT), calcification was found to be localized in the osteoarthritic cartilage (Fig. 1D). The collagen arrangement in the ECM of calcified cartilage was disordered. Abundant calcification was detected in the OA group (fig. S2). At 8 weeks, collagen banding disappeared and calcification appeared profusely on the surface of the collagen fibrils, as seen in unstained transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM) (figs. S3 and S4). These results indicate that cartilage calcification occurs early in OA and aggravates as the condition deteriorates.

Calcified EVs are involved in calcification of osteoarthritic cartilage
To further explore the pathomechanism of cartilage calcification in OA, ultrastructure and elemental analysis of control and osteoarthritic cartilage were carefully examined. Magnified SEM images of the cartilage surface showed many spherical particulates in the vicinity of the collagen fibrils in the second week osteoarthritic cartilage (Fig. 2A, red rectangles). Elemental analysis indicated that they contain both calcium and phosphate (Fig. 2B). In the 8-week
osteoarthritic cartilage, the particulates aggregated into larger calcific plaques and were distributed along the collagen fibrils (Fig. 2, A and C). The particulates and cartilage calcification could not be seen in any cartilage samples from control groups (Fig. 2A and fig. S5). The relative amount of calcium and phosphorus in the cartilage of both OA groups was much higher than those of the controls (Fig. 2D). In contrast, the relative amount of carbon and oxygen in the osteoarthritic cartilage was much lower than the control (fig. S6). Within the fibrous layer of the cartilage, calcified particulates were identified around the collagen fibrils. The extent of aggregation and

![Image of SEM images showing calcified EVs in control and OA cartilage]

**Fig. 2. Calcified EVs are involved in calcification of osteoarthritic cartilage.** (A) SEM images of the cartilage from control and osteoarthritic groups. Scale bar, 5 μm. High magnification of the area depicted by the red rectangles showed calcified particulates and mineralized deposits (arrows). Scale bars, 200 nm. (B and C) Elemental analysis of the calcified particulates indicated by the red arrow in (A). (D) Quantitative analysis of element distribution in the cartilage (n = 6). ns, no significance. *P < 0.05, **P < 0.01, and ***P < 0.001. (E) (i) Representative TEM image of the cartilage from the 2-week control group. Particulates were observed along the collagen fibrils (arrows). Scale bar, 200 nm. (ii and iii) Representative TEM images of the cartilage from the 2-week OA group. Calcified particulates (arrows). Scale bar, 500 nm. The amorphous nature of the electron-dense calcium phosphate in the calcified particulates (arrows) was validated by selected area electron diffraction (inset). (F) Elemental analysis of the selected area in (E-iii). (G) Elemental mapping of the calcified particulates (arrows). Scale bar, 500 nm. (H) Representative TEM images of EVs extracted from the control and OA cartilage. Scale bars, 300 nm. High magnification of the EVs outlined with the red rectangles in the low magnification images. Scale bars, 100 nm. (I) Nanoparticle-tracking analyses (NTAs) of the EVs from the control and OA cartilage.
Compared to the EVs derived from the control cartilage, the EVs control and OA rats were isolated. The representative cup shape of calcified EVs found in SEM and TEM, the EVs from cartilage of the existence of calcified EVs in OA group. To verify the existence OA group were replaced by ample mineral deposits in the ECM in and oxygen (Fig. 2G). The calcified particulates seen in the 2-week (Fig. 2, E-iii and F) were calcified, containing calcium, phosphorus, and oxygen (Fig. 2G). The calcified particulates seen in the 2-week OA group were replaced by ample mineral deposits in the ECM in the 8-week osteoarthritic specimens (fig. S11). Therefore, we speculated the existence of calcified EVs in OA group. To verify the existence of calcified EVs found in SEM and TEM, the EVs from cartilage of control and OA rats were isolated. The representative cup shape appearance of the EVs was obvious in both control and OA group. Compared to the EVs derived from the control cartilage, the EVs from osteoarthritic cartilage were larger in size and acquired an electron-dense internal structure as calcium phosphate nucleation occurred (Fig. 2, H and I, and fig. S12). Collectively, these results are indicative of the contribution of calcified EVs to cartilage calcification in OA of the TMJ.

**Calcified EVs are derived from mineral-bearing autophagosomes**

To clarify the origin of calcified EVs, chondrocyte autophagy and its relationship to calcified EVs were investigated. The results showed that the levels of LC3 protein were up-regulated in the cartilage from OA groups comparing to those of the controls (Fig. 3, A and B, and fig. S13). However, unlike autophagosomes in the control group that fused with lysosomes, autophagosome-lysosome fusion in the osteoarthritic chondrocytes was reduced (Fig. 3, C and D, and fig. S14). Gene expressions of autophagosome-lysosome fusion-related factors [SNAP29 (Synaptosome Associated Protein 29), STX17 (Syntaxin 17) and VAMP8 (Vesicle Associated Membrane Protein 8)] were significantly down-regulated in the OA groups comparing to the controls (Fig. 3E). In addition, the autophagosomes in the OA chondrocyte contained an amorphous mineral of low crystallinity with Ca and P elements, while autophagosomes in control chondrocytes did not (Fig. 3, F to I). These results indicate that membrane fusion between autophagosomes and lysosomes is inhibited in the osteoarthritic chondrocytes and that the release of calcified EVs may be attributed to the blockage of autophagic flux and accumulation of autophagosomes.

**Autophagic LC3⁺ EVs are involved in calcification of collagen hydrogels**

To further examine the contribution of autophagy and calcified EVs to pathological cartilage calcification, an in vitro model was developed by coculturing of three-dimensional (3D)–type II collagen hydrogel with primary chondrocytes to visualize the mineralization process (figs. S15 and S16). Alizarin Red S staining indicated that chondrocytes cultured in calcified medium induced calcification of the type II collagen, whereas those cultured in control medium did not (Fig. 4, A to E, and fig. S17). In the present in vitro system, chondrocytes cultured in calcified medium had osteoarthritic changes, including increased gene and protein expression of matrix metalloproteinase 3 (MMP-3) and decreased expression of aggrecan comparing to the controls (all \( P < 0.05 \)) (fig. S18). Treatment of the calcified chondrocytes with wortmannin, a pharmacological agent used for blocking autophagosome formation (28), resulted in inhibition of calcification (Fig. 4, A to E). Similar to what was observed in vivo in the osteoarthritic cartilage (Fig. 2A), small calcifications entrapped by the collagen matrix fused to form larger calcific plaques (Fig. 4B), and the LC3⁺ EVs in the 3D culture system were visualized to aggregate to produce spherical calcified structures (Fig. 4C). There were more LC3⁺ EVs in 3-day calcified group than the 7-day or the 14-day calcified groups (Fig. 4, C and F). In the 14-day calcified group, the calcifications aggregated along the collagen fibrils and the LC3⁺ EVs almost disappeared (Fig. 4, C and F, and fig. S19). These results confirmed that autophagic LC3⁺ EVs derived from the chondrocytes are involved in the calcification of the type II collagen hydrogel.

**Secretory autophagosomes are the origin of calcified EVs**

When chondrocytes that had been cultured for 7 days were examined by TEM, the EVs in the control group did not contain Ca and P (Fig. 5A), whereas EVs in calcified group contained amorphous calcium and phosphorus and therefore were coined calcified EVs (Fig. 5, B to D, and fig. S20). In calcified group, the calcified EVs were observed within the chondrocytes (Fig. 5E) and in the ECM (Fig. 5F and fig. S21). LC3 immuno-TEM showed that in the control group, mineral granules were absent and LC3 was located along the periphery of the calcified EVs. However, in the calcified group, the calcifications aggregated along the periphery of the calcified EVs, which located closely to the mineral granules (Fig. 5, G and H), and LC3⁺ calcified EVs aggregated to produce calcified nodules within the ECM (Fig. 5I and fig. S23). The mineral within the calcified EVs was confirmed to be Ca, P, and O (Fig. 5I), and the calcified EVs aggregated to produce calcified plaques (Fig. 5K and fig. S24). These data suggest that secretory autophagosomes from chondrocyte are the origin of calcified EVs.

**LC3⁺ calcified EVs initiate calcification of collagen hydrogel**

The characteristic and function of calcified EVs were further examined. The EVs from chondrocytes of control group appeared round and double-membrane structures. Those from the calcified group were larger and contained electron-dense materials (Fig. 6A). Size distribution of the EVs from the calcified group (Fig. 6B) was similar to those seen in vivo in the osteoarthritic cartilage (Fig. 2I). Protein expression of EVs specific makers (Alix, CD9, and Tsg101) was verified in the control and calcified EVs (Fig. 6C), and the relative expression of LC3B/CD9 on the calcified EVs was higher than that of the control EVs (Fig. 6, C and D). In addition, when chondrocytes were cultured in the calcified medium for 48 hours, their calcified EVs showed considerable aggregation (Fig. 6E) and could induce obvious collagen calcification (Fig. 6F and fig. S25). The number of EVs released per cell was significantly higher in the control group than that of the calcified, while it decreased after wortmannin treatment (Fig. 6G).

Flow cytometric analysis was further used to clarify the autophagy-derived calcified EVs among the total EVs from chondrocytes. The results showed that the percentage of LC3⁺ EVs was 50.79% among the total CD9⁺ EVs. Autophagy augment by rapamycin increased above ratio to 79.27%, while autophagy inhibition by wortmannin decreased the ratio to 6.49% (Fig. 6, H and I). In addition, the percentage of LC3⁺ (Ca²⁺)⁺ EVs in the calcified group was 54.57% among the total (Ca²⁺)⁺ EVs, which is indicative of the capability of LC3⁺ EVs to transport Ca²⁺. Similarly, autophagy promoting triggered the increase release of LC3⁺ (Ca²⁺)⁺ EVs to as high as 81.58%,
while autophagy inhibiting decreased the release of LC3\(^+\) (Ca\(^{2+}\))\(^+\) EVs to 1.68% (Fig. 6, H and I). However, the percentages of LC3\(^+\) (Ca\(^{2+}\))\(^+\) EVs were around 98% among total LC3\(^+\) EVs in all groups and did not exhibit any difference (Fig. 6I). These results verified that LC3\(^+\) calcified EVs increased extensively upon calcification of the chondrocytes, and the process was controlled by autophagy.

The calcified EVs could induce obvious calcification of type II collagen, while EVs from control group or from wortmannin-treated calcified group could not (Fig. 6J). Of note, about 44.28% of the EVs were LC3\(^-\) (Ca\(^{2+}\))\(^+\) in the calcified group. To clarify the function of this group of EVs in pathological calcification, the LC3\(^+\) EVs from the calcified group were removed with LC3-conjugated magnetic microbeads. The rest of the EVs were incubated with the collagen hydrogel. Both TEM and SEM showed that there was very little calcification after the LC3\(^+\) EVs were removed (Fig. 6J). This indicates that LC3\(^+\) calcified EVs initiate pathological calcification in OA.

**Release of LC3\(^+\) calcified EVs results from HDAC6-induced microtubule destabilization**

Similar to what was observed in vivo in the osteoarthritic cartilage (Fig. 3, C and D), colocalization of LC3 and LAMP1 (Lysosomal Associated Membrane Protein 1) (Fig. 7, A and C) and gene expressions of autophagosome-lysosome fusion-related factors (fig. S26)
Fig. 4. LC3+ calcified EVs are involved in calcification of collagen hydrogels. (A) Alizarin red S staining of chondrocytes cultured in type II collagen hydrogel for 3, 7, and 14 days. Control, control medium; calcified, calcified medium; calcified + wort, calcified medium with wortmannin (100 nM). Scale bar, 2 mm. (B) Immunofluorescence microscopy of chondrocytes cultured in type II collagen hydrogel for 7 days. Alizarin Red S fluorescence indicates calcification within the hydrogels. Type II collagen, green; DAPI, blue. Scale bar, 10 μm. (C) Bright-field and immunofluorescence images showing the presence of LC3+ calcified EVs in type II collagen hydrogel that were cultured in calcified medium. Calcification in the hydrogel was indicated by the arrows. Scale bar, 50 μm. (D) Quantitative analysis of the area occupied by Alizarin Red S–stained calcific regions in the different groups in (A) (n = 6). (E) Quantitative analysis of the relative fluorescence area of Alizarin Red S in the different groups in (B) (n = 6). (F) Quantitative analysis of the relative fluorescence areas of LC3 in the different groups in (C) (n = 6). ***P < 0.001 and **P < 0.01 and ns < 0.05.
were significantly decreased in the chondrocytes from 7-day calcified group comparing to those of the controls, indicating the blockage of autophagic flux and accumulation of autophagosomes within chondrocytes in calcified situation. To verify this observation, monomeric red fluorescent protein (mRFP)–green fluorescent protein (GFP)–LC3 adenovirus transfection was performed to investigate the fate of autophagolysosomes in the chondrocytes. As shown in Fig. 7D, autophagosomes were abundant in chondrocytes that were cultured in calcified medium, and there was almost no fusion between the autophagosomes and the lysosomes (Fig. 7E).

The fusion of autophagosomes with lysosomes was regulated by microtubule stabilization, which was modulated by histone deacetylase 6 (HDAC6)–induced α-tubulin deacetylation (29, 30); thus, the structure and acetylation of α-tubulin, as well as HDAC6 activity, were significantly decreased in the chondrocytes from 7-day calcified group comparing to those of the controls, indicating the blockage of autophagic flux and accumulation of autophagosomes within chondrocytes in calcified situation. To verify this observation, monomeric red fluorescent protein (mRFP)–green fluorescent protein (GFP)–LC3 adenovirus transfection was performed to investigate the fate of autophagolysosomes in the chondrocytes. As shown in Fig. 7D, autophagosomes were abundant in chondrocytes that were cultured in calcified medium, and there was almost no fusion between the autophagosomes and the lysosomes (Fig. 7E).
were examined to evaluate the stability of microtubules (31, 32). The results showed that chondrocytes in the calcified group exhibited disordered and decreased α-tubulin expression (Fig. 7, F and G), decreased level of acetylated tubulin (Fig. 7, H and I), and increased level of HDAC6 activity (Fig. 7J), respectively, comparing to those of the controls. Thus, inhibition of autophagic flux and accumulation of autophagosomes contribute to the release of LC3⁺ calcified EVs from the chondrocytes into the ECM. Such a process, in turn, is related to HDAC6-induced microtubule destabilization.

Inhibition of HDAC6 reverses osteoarthritic cartilage calcification

Because HDAC6 inhibitor tubacin has been proved to recover the fusion of autophagosomes with lysosomes (autophagic flux) by...
restoration of the microtubule structure (32), we investigated the role of LC3\(^+\) calcified EVs in osteoarthritic condylar cartilage calcification and degradation by intra-articular injection of tubacin. Consistent with the above results, flow cytometry showed that there was a higher percentage of LC3\(^+\) calcified EVs in the OA group than the sham group, while HDAC6 inhibition decreased the percentage significantly (Fig. 8A).

Cartilage degradation was evident in the osteoarthritic rats, which was manifested by cartilage thinning, loss of proteoglycans, and abnormal cartilage calcification, while cartilage calcification and degradation were obviously attenuated by the tubacin treatment, evidenced by OARSI histological score and significant increase of cartilage thickness and proteoglycan areas as well as decrease of calcified cartilage area (Fig. 8B and fig. S27). Results of TEM and SEM further verified that tubacin treatment rescued the disorganized and fragmented collagen fibrils, the calcified deposits in the cartilage ECM, and cartilage degeneration (Fig. 8C).

**DISCUSSION**

Although pathological cartilage calcification almost occurs in all osteoarthritic joints, its initiating factor is unclear (4). Calcified EVs function as foci for the crystallization of calcium phosphate during developmental endochondral ossification (33). However, the origin of calcified EVs and their effects on pathological cartilage calcification are unknown. In the present study, a rat TMJ-OA model was used initially to investigate the correlation between calcified EVs and secretory autophagosomes and their roles in pathological cartilage calcification in vivo. Pathological cartilage calcification was found to occur early in OA of the TMJ. In addition, LC3\(^+\) calcified EVs were detected in the affected cartilage.

Captivated by these in vivo observations, chondrocytes were cultured ex vivo in type II collagen hydrogel to further substantiate the in vivo results. Because chondrocytes in the present in vitro system had osteoarthritic changes, such as increased expression of MMP-3...
and decreased expression of aggrecan (fig. S18), we examine the contribution of calcified EVs to pathological cartilage calcification by the in vitro systems. Calcified EVs released by chondrocytes were LC3+ and critical for inducing calcification. Secretion of the LC3+ calcified EVs was caused by inhibition of autophagy flux via HDAC6-mediated microtubule destabilization. Blocking the release of calcified EVs in vivo by an HDAC6 inhibitor effectively reversed pathological cartilage calcification and the progression of OA. Together, the

**Fig. 8. Inhibition of HDAC6 activity reverses cartilage calcification and degeneration in OA.** (A) Flow cytometry of LC3 and Ca2+ on EVs and the corresponding quantification of percentage of the mean fluorescence intensity of EVs derived from 8-week sham control (Sham), OA, and OA + tubacin (tub) groups (n = 6). (B) Von Kossa and hematoxylin and eosin staining, immunofluorescence, and safranin O staining of central TMJ sections in different groups. Corresponding quantification of percent area of the calcified region and proteoglycan area are shown in the right (n = 6). The areas (between the white lines) indicate calcified region within the cartilage. Scale bars, 100 μm. (C) TEM and SEM images of the hypertrophic layer of the TMJ condylar cartilage in different groups. *P < 0.05, **P < 0.01, and ***P < 0.001.
present work identified a previously unrecognized mechanism that calcified EVs derived from autophagosomes are responsible for initiating pathological cartilage calcification.

Vesicles associated with calcification were identified in the matrix of epiphyseal cartilage during the late 1960s and early 1970s. Those vesicles vary considerably in size and shape (34–36). They are derived from chondrocytes during endochondral ossification and provide a suitable microenvironment for initiating apatite deposition (37). Mineralization mediated by calcified EVs is an orchestrated sequence of ultrastructural and biochemical events that leads to crystal nucleation and growth (15). Proteins on the membranes of EVs such as nucleoside triphosphate pyrophosphohydrolase, multi-pass inorganic pyrophosphate transport regulator, tissue-nonspecific alkaline phosphatase, and annexin arbitrate the influx of Ca\(^{2+}\) and PO\(_4^{3-}\) into the EVs (38). In the present study, calcified EVs derived from osteoarthritic cartilage were isolated and characterized using multiple approaches. These calcified EVs were heterogeneous in size, with diameters between 200 and 300 nm, and were closely related to osteoarthritic calcification. Similar to OA, calcified EVs are released during atherosclerotic artery calcification by vascular SMCs; those calcified EVs penetrate the intima media and contribute potentially to atherothrombosis (39, 40). To classify the EVs in our in vivo model, we have isolated the EVs from cartilage of control and OA rats and further characterized them with nanoparticle-tracking analyses (NTAs) and TEM. Further studies are needed to explore the in situ characteristic of the EVs in OA cartilage by the immunogold labeling in the TEM. TEM images of EVs isolated from the in vitro systems revealed an electron dense structure, and their hydroxyapatite-associated structures were in accordance with the calcified EVs (41). Further study is needed to verify above finding by elemental mapping of isolated EVs. In addition, our result of Alizarin Red S staining also verified that the calcification of ECM could not be observed in the present calcified medium without the chondrocytes (fig. S17).

High-angle annular dark-field–STEM energy-dispersive x-ray spectroscopy (EDS) elemental mapping (fig. S23) further demonstrated that the calcification of the ECM contains nitrogen from organic matter, supporting the notion that the calcification is not derived from the precipitation of supersaturated calcium and phosphate (42). Regulation of calcification is complex and requires the coordinated actions of calcification inhibitors and propagators. Besides the production of the calcified EVs, pathological calcification also proceeds when this equilibrium is disrupted by factors that include changes in the structure and composition of ECM such as collagen and proteoglycans, changes in extracellular Ca\(^{2+}\) levels, dysregulation of pyrophosphate and phosphate metabolism, and imbalance between inhibitors and promoters in noncollagenous proteins (4).

Although the pathogenic roles of calcified EVs in calcification have been established, the mechanism for their production remains largely unknown. EVs are purportedly produced by outward budding of the plasma membrane or by an intracellular endocytic trafficking pathway that involves fusion of multivesicular late endocytic compartments (multivesicular bodies) with the plasma membrane (43). Nevertheless, the most discussed mechanism of calcified EV formation is budding from the plasma membrane. A study that compared the lipid and protein composition of EVs and the plasma membrane of chondrocytes reported that these entities have a similar composition (10). In physiological mineralization processes, epiphyseal chondrocytes and bone osteoblasts were shown to release EVs from budding at specific sites of their plasma membrane. Electron microscopy also demonstrated that calcified EVs derived from SMCs were present in vascular calcifications of patients with chronic kidney disease or atherosclerotic lesions (44). Recent work has shown that SMC-derived calcified EVs originated from an exosomal pathway. These calcified EVs were processed through multivesicular bodies. Inhibition of exosomal pathway in vascular SMCs inhibited their ability to mineralize (45). However, little is known about the relationship between the calcified EVs and secretory autophagy. In the present work, it was found that calcified EVs in osteoarthritic cartilage encapsulate autophagy-related proteins such as LC3 and have sizes in the range of the autophagosomes. Ex vivo experiments also demonstrated that chondrocytes released LC3\(^{3+}\) calcified EVs, which, in turn, induced calcification of the type II collagen hydrogel. The percentage of LC3\(^{3+}\) (Ca\(^{2+}\))\(^{3+}\) EVs in calcified group was significantly inhibited by wortmannin, while rapamycin significantly up-regulated the release of LC3\(^{3+}\) calcified EVs. These observations suggest autophagic control of calcified EV release. Furthermore, calcification was inhibited when the LC3\(^{3+}\) EVs were cleared by magnetic beads. This indicates that LC3\(^{3+}\) calcified EVs play the major role in inducing pathological calcification in OA, and such property of LC3\(^{3+}\) calcified EVs also helps to design and deliver some synthetic nanoparticle carriers to specifically target and clear the calcified EVs to disrupt the hydroxyapatite nucleation and attenuated the progression of OA more effectively.

Secretory autophagy is a mechanistically orchestrated process that plays roles in physiological and pathological conditions (46). Secretory autophagy uses components of the autophagy machinery to selectively transport and secrete cargoes out of the cell. Mitochondria, endoplasmic reticulum, and other organelles are reported to be associated with calcium and phosphorus handling, which may also be sequestered by autophagosomes (19). In the maintenance of cardiac homeostasis, cardiomyocytes eject dysfunctional mitochondria and other cargoes in discrete membranous particles that are reminiscent of neural exophers, a specific form of EVs. This is achieved through a process driven by the autophagy machinery that is up-regulated during cardiac stress (47). Secretory autophagy has also been implicated in extrusion of β-amyloid peptide aggregates associated with Alzheimer’s disease and the cytosolic cargo α-synuclein in Parkinson’s disease (21). Furthermore, in osteoblast-specific, autophagy-deficient mice, autophagy was found to be involved in mineralization of osteoblasts and in bone homeostasis (48). In the authors' previous study, it was hypothesized that mineralization precursors are transported via mitophagy, a specific form of autophagy, to the EVs during cell-mediated biominerallization (18). In the present study, increased expression of LC3 and failure of fusion of lysosomes with autophagosomes were observed in the osteoarthritic cartilage. These phenomena provided evidence of the interruption of autophagy flux (49). We showed that wortmannin significantly decreased the release of autophagic LC3\(^{3+}\) calcified EVs (Fig 4, A and B). Because wortmannin is a phosphatidylinositol 3-kinase (PI3K) inhibitor and regulates the autophagosome formation through interfering with the PI3K/Akt signaling pathway (50), further study is needed to explore the effects of PI3K/Akt signaling pathway on the secretion of autophagic LC3\(^{3+}\) calcified EVs. The present study also found that disruption of the intracellular microtubular arrangement during chondrocyte calcification prevents fusion of the lysosomes with the autophagosomes. Nevertheless, disruption of autophagy may cause apoptosis or necrosis of the chondrocytes (51) that aggravate cartilage degeneration. Thus, the role of autophagy
in pathological calcification should be examined in detail in future studies.

Microtubules are important components of the cytoskeleton and perform essential functions such as positioning of organelles and fusion of autophagosomes with lysosomes. The microtubules are regulated by an extensive array of reversible posttranslational modifications in which tubulin acetylation has been implicated in regulating microtubule stability and function (30). Unique among the HDAC family members, HDAC6 has intrinsic ubiquitin-binding activity and is associated with deacetylation of α-tubulin in vitro and in vivo (31). Overexpression of HDAC6 induced the expression of deacetylated α-tubulin and thereafter microtubules destabilization (32). Therefore, the structure and acetylation of α-tubulin and HDAC6 activity have been widely used to evaluate the stability and function of microtubules (31, 32). The present study showed that HDAC6 activity was enhanced in pathological calcification, which subsequently caused microtubule destabilization due to the reduction of acetyl-α-tubulin. Accordingly, tubacin, a highly potent and selective HDAC6 inhibitor, was used as the regulator of the LC3+ calcified EVs in the animal experiment. Tubacin was found to potently inhibit the release of autophagic calcified EVs and prevent the progression of OA. To our knowledge, this is a study that uses microtubule intervention to suppress endogenous EVs as a therapeutic measure for OA. Such a strategy holds promise for future translational applications. However, further studies are needed to explore the detailed mechanism of microtubule destabilization of osteoarthritic chondrocytes.

In the progression of OA, pathological calcification in the affected joint is an important feature, and the pathogenic roles of these crystallites in the pathogenesis and progression of OA have been well studied. The present TEM results showed that the pathological calcification in the noncalcified layers (fibrous and proliferative layers) of OA cartilage were local and punctiform (figs. S7 and S8), while calcification in the calcified zone of OA cartilage was mass-like (fig. S2). Because of the limit of resolution (1 μm), the conventional light microscopy could not detect the pathological calcification in noncalcified articular cartilage (52) but only exhibited the expansion of the calcified cartilage zone. Therefore, pathological calcification of OA cartilage includes both punctiform calcification in noncalcified zone and large clumps in the calcified zone, which were in accordance to our previous reports (25, 26). In addition, the main pathological change of subchondral bone in OA is the abnormal bone remodeling (uncoupled bone resorption and formation) (25, 53, 54). The increased calcification in OA subchondral bone observed in the present study might due to the uncoupled bone remodeling, which was caused by the formation of mesenchymal stem cell clusters induced by high concentrations of active transforming growth factor–β in the local areas (53). It is suggested that pathological calcification might be a disease initiator instead of an outcome of OA progression (4). Inhibiting pathological crystallite deposition within joint tissues therefore represents a potential therapeutic target in the management of OA (4). However, to date, there is a lack of good in vitro models in this field of research. 3D culture of chondrocytes within a type II collagen hydrogel was used in the present work to recapitulate pathologically relevant microenvironmental conditions in OA. Pathological calcification is a common comorbidity in diseases such as atherosclerosis, aortic valve stenosis, diabetes, renal failure, and chronic inflammation (55, 56), and this innovative approach may be also used as a model for investigation of heterotopic calcification in tendon, ligament, and vessels.

In conclusion, we have demonstrated that LC3+ calcified EVs derived from the autophagosomes initiate pathological cartilage calcification in OA. Our findings present a novel pathophysiological mechanism for OA that may be used as a therapeutic target for treatment of OA (Fig. 9).

Fig. 9. Schematic diagram showing the synopsis of the findings. During OA progression, secretory autophagy is the origin of LC3-conjugated calcified EVs by osteoarthritic chondrocytes. The secretion of calcified EVs by osteoarthritic chondrocytes is caused by autophagic flux inhibition resulting from HDAC6-induced α-tubulin deacetylation and thereafter microtubular destabilization. When secreted to the ECM, the LC3-conjugated calcified EVs form the mineral nodule to initiate calcification of the osteoarthritic cartilage.
### MATERIALS AND METHODS

**Group designation and experimental scheme** are described in fig. S28.

**Rat OA model**

Female Sprague-Dawley rats (6 weeks old; weight, 140 to 160 g) were provided by the animal center of the Fourth Military Medical University. All experimental protocols met the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. A unilateral anterior crossbite procedure was used to induce osteoarthritic-like changes in the TMJ cartilage of rats (23). Control rats were subjected to the same procedure but without fixation of a metal tube. In the experimental rats, no differences were noted in the manifestation of osteoarthritic phenotype between the right and left TMJ cartilage.

**TMJ injection**

Each rat was laid sidewise after deep anesthesia with 1% intraperitoneal sodium pentobarbital. The needle with a custom-designed Hamilton-type syringe was inserted just below the zygomatic arch between the corner of the eye and ear until the outer surface of the mandibular ramus was reached. The orientation of the needle head was adjusted to enable it to slide along the bone wall to reach the TMJ region. A total of 50 μl of 100 nM tubacin (S1039, Selleck, Pittsburgh, PA, USA) diluted in phosphate-buffered saline (PBS) was injected locally into the TMJ region of the injection groups two times per week (23).

**Histochemical and immunofluorescence staining**

Pentobarbital overdose was performed to euthanize all rats. For each rat, a tissue block that included the joint capsule and 3 to 4 mm of the tissue surrounding the condyle was harvested. After fixation in 4% paraformaldehyde for 24 hours and 30% sucrose for 3 days, the specimens were embedded in optimal cutting temperature compound (Leica, Wetzlar, Germany) and stored at −80°C. The tissue was cut into 5-μm-thick sections. A cryofilm (Section-lab Co. Ltd., Hiroshima, Japan) was mounted onto the cut surface, and the specimen was tightly adhered to the cryofilm. For hematoxylin and eosin and Von Kossa staining, the cryofilm was stained with silver nitrate under the ultraviolet light for 2 min and washed with running water. The sections were stained with hematoxylin for 3 min and water-soluble eosin. For Alizarin Red S staining, the cryofilms were stained with Alizarin Red S (40 mM, pH 4.2; MilliporeSigma, Burlington, USA) for 20 min. The nuclei were counterstained with Hoechst 33342 (MilliporeSigma).

For immunofluorescence staining, the cryofilms were blocked with 1.5% goat serum (MilliporeSigma) and incubated with primary antibodies. The primary antibodies used were anti-LC3B (diluted at 1:200; 83506, Cell Signaling Technology, Danvers, MA, USA), anti-LAMP1 (diluted at 1:100; ab24170, Abcam, Cambridge, UK), anti-Col2 (diluted at 1:100; ab34712, Abcam), anti-α-tubulin (diluted at 1:200; 3873, Cell Signaling Technology), anti-aggrecan (diluted at 1:200; ab36861, Abcam), and anti–MMP-3 (diluted at 1:50; sc6839, Santa Cruz Biotechnology, TX, USA). The sections were incubated with secondary antibody (US Everbright Inc., Suzhou, China). After incubation, the sections were washed with PBS, and the nuclei were counterstained with Hoechst 33342.

For safranin O/fast green staining, the tissue blocks were embedded in paraffin after fixation in 4% paraformaldehyde for 4 hours and decalcified in 10% EDTA for 4 weeks. Serial sections (4 μm thick) were prepared through the TMJ in the sagittal plane. The sections were mounted on poly-L-lysine–coated glass slides. The mounted sections were deparaffinized and stained by 0.02% fast green for 5 min. They were rinsed with 1% acetic acid solution, stained in 0.1% safranin O solution for 5 min, and dehydrated with 95% ethyl alcohol, absolute ethyl alcohol, and xylene. To ensure reliable comparison between the specimens from different groups, the central sagittal sections of each joint were selected.

Photographs were taken by a confocal microscope (FV1000, Olympus, Tokyo, Japan), and the percentage area of condylar cartilage with pathological calcification, safranin O–positive area, and the relative fluorescence intensity were measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA) (54). The OARSI score in the different groups was evaluated according to the histological results (27).

**Micro–computed tomography**

Rat TMJs were scanned using a high-resolution micro-CT system (Inveon micro-CT system Siemens AG, Germany). Briefly, specimens were scanned at 80 keV and 500 mA. Two-dimensional slices with an 8-μm isotropic resolution were generated and used for 3D reconstruction of the TMJ specimen.

**SEM and EDS**

Cartilage specimens and glass slides containing collagen hydrogels and chondrocytes were fixed in 2.5% glutaraldehyde in phosphate buffer (0.01 M, pH 7.4), washed extensively with water, dehydrated with an ascending series of ethanol (30 to 100%), and treated with hexamethylsilane (Electron Microscopy Sciences, Hatfield, PA, USA) that was slowly evaporated. The dried specimens were secured to sample holders with carbon tape and imaged with a field-emission SEM (S-4800, Hitachi, Tokyo, Japan) operated at 5 kV. EDS (Element EDS System, Ametek, Berwyn, PA, USA) was used to characterize the mineral elemental composition in both the cartilage and calcific structures present within the collagen hydrogels.

**Chondrocyte isolation and cell culture**

Chondrocytes were isolated from the condylar cartilage of rat TMJs by digestion with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 20 min, followed by 0.2% type II collagenase (PC-50995, PlantChemMed Biology Co. Ltd. ShangHai, China) for 2 to 3 hours (57). Then, the chondrocytes were grown to around 90% confluency, followed by the replacement of control medium or calcified medium. The control medium consisted of Dulbecco’s modified Eagle medium/nutrient mixture F-12 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (PC-00001, PlanChemMed Biology Co.) and 1% penicillin-streptomycin solution (PC-86115, PlanChemMed Biology Co.). In calcified group, when cell growth reached about 90% confluency, cells were washed with PBS three times and then grown in the calcified medium consisted of control medium supplemented with 10 nM dexamethasone, 100 μM l-ascorbic acid, 1.1 mM calcium chloride, and 10 mM β-glycerol phosphate (all from MilliporeSigma). The respective medium was replaced every 3 days.

**Extracellular vesicle isolation**

For extracellular vesicle collection from medium, the cells were uniformly seeded in petri dishes at a density of 13.3 × 10^5/ml. After the designated culture period of chondrocytes (isolated from the condylar
cartilage) in control and calcified medium, the culture medium was substituted by complete medium containing EV-depleted FBS. The EV-depleted FBS was obtained by ultracentrifugation at 160,000g for 12 hours, which prevented contamination by FBS-derived EVs. After 24 hours, the supernatant was collected and subsequently centrifuged at 800g for 10 min and 2000g for 30 min. The EVs were isolated from the supernatant by centrifugation at 16,000g for 30 min and then washed twice with filtered PBS (58). At the designated supernatant collection time, the percentage of viable cells was more than 98%.

For collection of EVs from cartilage of control and OA groups, a tissue block that included the joint capsule and 3 to 4 mm of the tissue surrounding the condyle was harvested and maintained in PBS on ice before being immediately processed for EV isolation. The cartilage was separated and sliced into small pieces before being incubated with collagenase D and deoxyribonuclease I (all from MilliporeSigma) for 30 min at 37°C. A filtration step with a 0.70-μm pore-size filter was applied to remove the largest elements. The remaining liquid was differentially centrifuged at 800g for 10 min and 2000g for 30 min to remove cells and tissue debris. The supernatant was then further centrifuged at 16,000g for 30 min to collect the EVs, which were washed in PBS using the same ultracentrifugation conditions (58, 59).

The pellets of EVs from medium or cartilage were resuspended in 200 μl of PBS for NTA, electron microscope assays, flow cytometry, or used in coculture with type II collagen. The protein content of isolated EVs was measured using the bicinchoninic acid (BCA) Kit (Beyotime BioTech, China).

**Cell transfection with adenoviruses**

The cells were uniformly seeded in confocal dishes at a density of 3 × 10^5/ml (2 ml in each dish). Subsequent to the cells from different groups cultured for 7 days, they were transiently transfected with fluorescent mRFP (red)–GFP (green)–LC3 adenovirus (1.58 × 10^10 pfu/ml) according to the manufacturer’s instructions (Hanbio, Shanghai, China). Images were obtained using a confocal microscope (FV1000, Olympus, Tokyo, Japan).

**Alizarin Red S staining**

Cells from different groups were cultured for 3, 7, or 14 days. At the designated time point, the cells were stained with Alizarin Red S (40 mmol/liter, pH 4.2) after fixation with 10% formaldehyde. Excessive dye was removed after 30 min by washing with water. The plates were imaged with a Zeiss microscope (Thorn-wood, NY, USA). Stained areas were measured using the ImageJ software.

**Type II collagen isolation and hydrogel experiments**

As previously described, type II collagen was isolated from bovine tracheal cartilage (60). After delipidation, the cartilage was cut into small pieces and washed with 50 mM tris-HCl (pH 7.2), containing 25 mM EDTA-Na2, and 2 mM ethylmaleimide (all from MilliporeSigma). After incubation in 0.5 M acetic acid (pH 2.5), the collagen was digested by incubation in 0.5 M acetic acid (pH 2.5) containing 8 × 10^6 units pepsin/liter (MilliporeSigma). The extracted collagen was centrifuged (20,000g, 20 min) and dissolved in 0.5 M acetic acid (pH 2.5). Collagen hydrogels were prepared by slowly raising the pH of the solution to 7 to 8. The collagen formed a hydrogel network at this pH range. The neutralized collagen solution was added to the chambered wells and evaluated using SEM. Chondrocytes or EVs were added to the type II collagen network and incubated at 37°C for the indicated time period.

**Nanoparticle-tracking analyses**

Size distribution of the EVs was evaluated using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) equipped with a 405-nm laser. EVs collected from the medium or cartilage specimens were diluted in filtered PBS buffer to achieve a final concentration between 1 × 10^6/ml and 1 × 10^9/ml. The image of filtered PBS was taken to verify that the diluent contained no particle. A video of 60-s duration was taken with a frame rate of 30 frames/s, and particle movement was analyzed using NTA software (ZetaView 8.02.28).

**Transmission electron microscopy**

The cartilage specimens and chondrocyte-containing collagen hydrogels were fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4). The specimens were post-fixed in 1% osmium tetroxide, dehydrated in an ascending series of ethanol, immersed in propylene oxide, and embedded in epoxy resin. Ninety-nanometer–thick sections were obtained, stained with uranyl acetate and lead citrate and observed using a JEM-123 TEM (JEOL, Tokyo, Japan) at 110 kV.

For TEM observation of EVs, an EV pellet was resuspended in 200 μl of PBS and 20 μl of EVs were deposited on 200-mesh formvar-coated copper grids and dried at room temperature. The EVs were negatively stained with uranyl acetate before imaging.

**Elemental mapping**

Elemental mapping was performed using a STEM equipped with EDS. Specimens used for elemental mapping were not post-fixed with osmium tetroxide to avoid interference during EDS. Unstained thin sections were examined with a Technai G2 STEM (FEI, Hillsboro, USA) at 200 kV. Elemental mapping and selected area electron diffraction were conducted using an INCA x-sight detector (Oxford Instruments, Abingdon, UK). Mappings were acquired with the FEI TIA software using a spot dwell time of 300 ms with drift correction performed after every 30 images.

**Immuno-TEM**

Immuno-TEM was used for identifying LC3+ EVs. Chondrocytes cultured in collagen hydrogel were fixed in 2.5% glutaraldehyde, blocked with bovine serum albumin (MilliporeSigma), incubated with mouse anti-LC3B primary antibodies (diluted at 1:100; 83506, Cell Signaling Technology), and followed by incubation with gold-conjugated secondary antibodies (1:1500 dilution; 1.4-nm nanogold; Nanoprobes, Yaphank, NY, USA). The nanogold particles were silver-enhanced with HQ Silver (Nanoprobes). The specimens were then immersed in osmium acid, dehydrated in ascending ethanol series, immersed in propylene oxide, and embedded in epoxy resin. Sections (90 nm thick) were stained with uranyl acetate and lead citrate and observed using a TEM (Hitachi, H-600, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from the condylar cartilage of rats or chondrocytes using TriPure Isolation Reagent (11667165001, Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Complementary DNA was synthesized, and real-time polymerase chain reaction (RT-PCR) was performed in the manner previously reported (24). Quantitative RT-PCR (RT-qPCR) was performed with FastStart Universal SYBR Green Master reagent (Roche) and
primer pairs using an ABI Step One-Plus instrument (Applied Biosystems, Thermo Fisher Scientific). Gene expression levels were estimated by the $2^{-\Delta\Delta C_t}$ method with glyceraldehyde 3-phosphate dehydrogenase gene expression level as internal control. Primers sequences are presented in table S1.

**Western blot**
The EVs were mixed with lysis buffer (Beyotime BioTech, China) that was supplemented with a protease inhibitor cocktail (Roche). Total protein amount of the EVs was measured by the BCA Kit (Beyotime BioTech, China). Purified proteins were separated in 6, 10, or 12% SDS–polyacrylamide gel electrophoresis (120 V for stacking gel and 160 V for separation gel) and transferred to polyvinylidene fluoride membranes (MilliporeSigma). The membrane was blocked with 5% bovine serum albumin and incubated overnight with primary antibodies. The antibodies used were mouse anti-Alix (diluted at 1:1000; ab92726, Abcam, Cambridge, UK), mouse anti-CD9 (diluted at 1:1000; ab92726, Abcam, Cambridge, UK), mouse anti-TSG101 (diluted at 1:1000; ab125011, Abcam), rabbit anti-calnexin (diluted at 1:1000; 10427-2-AP, Proteintech, Rosemont, IL, USA), and mouse anti-CLC3B (diluted at 1:1000; 83506, Cell Signaling Technology). After treatment with the primary antibodies, the blotted membrane was incubated with secondary antibodies and visualized using a chemiluminescence detection system (Chemidoc, Bio-Rad, XRS, Hercules, CA, USA).

**HDAC6 activity**
Evaluation of HDAC6 activity was conducted according to the manufacturer’s instructions (#50076, BPS Bioscience, San Diego, CA, USA). Briefly, cell lysates of the chondrocytes in the collagen hydrogels were diluted in HDAC assay buffer and mixed with the substrate. The HDAC developer was added and incubated with the cell lysates. Enzyme activities were measured with a spectrofluorometer with excitation at a wavelength of 380 nm and detection of emitted light of 460 nm.

**Flow cytometry**
EVs derived from the chondrocytes in the collagen hydrogels and the cartilage specimens were isolated as previously described (59) and resuspended in 200 μl of PBS. The EVs were incubated with anti-CD9 antibody (diluted at 1:100; ab92726, Abcam), anti-CLC3B (diluted at 1:100; 83506, Cell Signaling Technology), and Ca$^{2+}$ fuel (diluted at 1:200; Fluo-4 AM ester, US Everbright, Suzhou, China) and mixed for 30 min. After the mixture was centrifuged at 12,000 rpm for 1 min, the pellet was resuspended in 2% serum albumin in PBS for incubation with secondary antibodies. After washing the EVs three times with 2% bovine serum albumin in PBS, CD9, LC3, and Ca$^{2+}$ detection was performed using a flow cytometer (CytoFLEX, Beckman Coulter, Brea, CA, USA). Data analysis was performed using the FlowJo 10.0 software (FlowJo LLC, Ashland, OR, USA).

**Coculture of EVs and collagen**
The collagen solution extracted from the cartilage specimens was dropped on a 400-mesh nickel TEM grid and air-dried. The collagen-coated grids were floated upside down over the EV solution (250 μg/ml) from different groups and imaged with TEM. For SEM, EVs subpopulations was performed with magnetic-activated cell sorting (58). Briefly, the EVs were incubated with fluorescein isothiocyanate (FITC)–conjugated anti-LC3 antibodies (NB100-2220F, Novus Biologicals, Littleton, CO, USA). This was followed by incubation with anti-FITC MicroBeads (130-048-701, Miltenyi Biotec, Cologne, Germany). The mixture was washed with PBS and passed through a column within a magnetic field. The flow-through fraction was defined as the negative fraction and added to the collagen network for subsequent experiments.

**Statistical analysis**
Analyses were performed using GraphPad Prism 8.0 (GraphPad Software, USA). All data were presented as means ± SDs. The Shapiro-Wilk test and modified Leven test were used, respectively, to test the normality and homoscedasticity assumptions of the corresponding data sets. The Student’s t test, one- or two-way analysis of variance (ANOVA) followed by Holm–Šidák multiple comparison tests was used to evaluate the differences among groups. For all tests, statistical significance was preset at α = 0.05.

**Magnetic enrichment of LC3⁻ EVs**
Enrichment of LC3⁻ EVs subpopulations was performed with magnetic-activated cell sorting (58). Briefly, the EVs were incubated with fluorescein isothiocyanate (FITC)–conjugated anti-LC3 antibodies (NB100-2220F, Novus Biologicals, Littleton, CO, USA). This was followed by incubation with anti-FITC MicroBeads (130-048-701, Miltenyi Biotec, Cologne, Germany). The mixture was washed with PBS and passed through a column within a magnetic field. The flow-through fraction was defined as the negative fraction and added to the collagen network for subsequent experiments.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abn1556

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**

1. N. Østerås, I. B. Blaker, T. Hjortland, E. Cottrell, J. G. Quicke, K. S. Dziedzic, S. Blackburn, A. Paulsen, Improving osteoarthritis management in primary healthcare: Results from a quasi-experimental study. *BMC Musculoskelet. Disord.* 22, 79 (2021).
2. J. M. Pelletier, A. J. Barr, F. M. Cicuttini, C. Cooper, M. B. Goldring, S. R. Goldring, G. Jones, J. T. Jeitchal, J. Pelletier, Osteoarthritis. *Nat. Rev. Dis. Primers.* 2, 16072 (2016).
3. E. G. J. Ripmeester, U. T. Timur, M. M. J. Caron, T. J. M. Welting, Recent insights into the contribution of the changing hypertrophic chondrocyte phenotype and fibrocartilage degeneration. *Arthritis Res. Ther.* 20, 104 (2018).
4. J. F. Yan, W. P. Qin, B. C. Xiao, Q. Q. Wan, F. R. Tay, L. N. Niu, K. Jiao, Pathological calcification in osteoarthritis: An outcome or a disease initiator? *Biol. Rev. Camb. Philos. Soc.* 95, 960–985 (2020).
5. T. Hlaweckel, J. Hubert, S. Hischke, M. Krause, J. Bertrand, B. C. Schmidt, A. Kronz, K. Püschel, W. Rüther, A. Niemeier, Calcification of the acetaldehyde labrum of the hip: Prevalence in the general population and relation to hip articular cartilage and fibrocartilage degeneration. *Arthritis Res. Ther.* 20, 33–50 (2018).
6. J. Hubert, L. Weiser, S. Hischke, A. Uhlig, T. Rolvien, T. Schmidt, S. K. Butscheidt, K. Püschel, W. Lehmann, F. T. B. habil, T. Hlaweckel, Cartilage calcification of the ankle joint is associated with osteoarthritis in the general population. *BMC Musculoskelet. Disord.* 19, 169 (2018).
7. T. Hlaweckel, J. Hubert, S. Hischke, T. Rolvien, M. Krause, K. Püschel, W. Rüther, A. Niemeier, Microcalcification of lumbar spine intervertebral discs and facet joints is associated with cartilage degeneration, but differs in prevalence and its relation to age. *J. Orthop. Res.* 35, 2692–2699 (2017).
8. Y. Sun, A. M. Franklin, D. R. Mauhehan, E. N. Hanley, Biological effects of phosphocitrate on osteoarthritic articular chondrocytes. *Open Rheumatol.* 11, 62–74 (2017).
9. S. Ansari, B. W. M. Wildt, M. A. M. Vis, C. E. Korte, K. Ito, S. Hofmann, Y. Y. U. Y. Maxx, Matrix vesicles: Role in bone mineralization and potential use as therapeutics. *Pharmaceuticals (Basel)* 14, 289 (2021).
10. M. Bottini, S. Mebarek, K. A. Anderson, S. A. Kiliszek, L. Bozycki, M. Bolean, P. Ciancaglini, J. B. Pikula, S. Pikula, D. Magne, N. Volkman, D. Hanein, J. L. Millán, R. Buchet, Matrix vesicles from chondrocytes and osteoblasts: Their biogenesis, properties, functions and biomimetic models. *Biochim. Biophys. Acta Gen. Subj.* 1862, 532–546 (2018).
11. H. C. Anderson, Electron microscopic studies of induced cartilage development and calcification. *J. Cell Biol.* 33, 81–101 (1967).
12. E. Bonucci, Fine structure of early cartilage calcification. *J. Ultrastruct. Res.* 20, 33–50 (1967).
M. Ponnuswak, M. A. Mandell, T. Kimura, S. Chauhan, C. Cleyrat, V. Deretic, Secretory autophagy. *Curr. Opin. Cell Biol.* **35**, 106–115 (2016).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Cell* **176**, 11–42 (2019).

J. D. Hutcheson, C. Goettsch, T. Pham, M. Iwashita, M. Aikawa, S. A. Singh, E. Aikawa, Autophagy and its relevance in metabolic and degenerative disease. *Front. Endocrinol (Lausanne).* **3**, 266 (2020).

E. Golub, Biomineralization and matrix vesicles in biology and pathology. *Semin. Immunopathol.* **33**, 409–417 (2011).

B. Levine, G. Kroemer, Biological functions of autophagy genes: A disease perspective. *Nat. Rev. Mol. Cell Biol.* **17**, 671–680 (2016).

P. Ejlerskov, I. Rasmussen, T. T. Nielsen, A. Bergström, Y. Tohyama, P. H. Jensen, F. Vilhardt, P. MacMullan, G. McMahon, G. McCarthy, Detection of basic calcium phosphate crystals in isolated rat hepatocytes. *Angew. Chem. Int. Ed. Engl.* **41**, 938–942 (2002).

M. Wang, Unilateral anterior crossbite induces aberrant mineral deposition in degenerative temporomandibular joint. *Adv. Sci.* **5**, e1800873 (2018).

J. Debnath, The LC3-conjugation machinery specifies the loading of RNA-binding proteins into extracellular vesicles. *Nat. Cell Biol.* **18**, 289–302 (1997).

C. D. Gonzalez, R. Resnik, M. I. Vaccaro, Secretory autophagy and its relevance in metabolic and degenerative disease. *Front. Endocrinol (Lausanne).* **3**, 266 (2020).

C. J. Williams, The role of ANKH in pathologic mineralization of cartilage. *Rheumatol.** 28**, 145–151 (2016).

P. MacMullan, G. McMahon, G. McCarthy, Detection of basic calcium phosphate crystals in isolated rat hepatocytes. *Angew. Chem. Int. Ed. Engl.* **41**, 938–942 (2002).

K. Jiao, M. Zhang, L. Niu, S. Yu, Y. Li, J. Liu, W. Zhang, HDAC6-mediated α-tubulin deacetylation suppresses autophagy and enhances motility of podocytes in diabetic nephropathy. *J. Cell Mol. Med.* **19**, 1548–1580 (2015).

J. D. Hutcheson, C. Goettsch, S. Bertazzo, N. Maldonado, J. L. Ruiz, W. Goh, K. Yabusaki, J. T. Faits, C. Bouten, G. Franck, T. Quillard, P. Libby, M. Aikawa, S. Weinbaum, E. Aikawa, Genetics and growth of extracellular vesicle-derived microcalcification in atherosclerotic plaques. *Nat. Mater.* **15**, 335–343 (2016).

M. Ponnuswak, M. A. Mandell, T. Kimura, S. Chauhan, C. Cleyrat, V. Deretic, Secretory autophagy. *Curr. Opin. Cell Biol.* **35**, 106–115 (2016).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

P. Eilerskov, I. Rasmussen, T. T. Nielsen, A. Bergström, T. Pham, M. Iwashita, M. Aikawa, S. A. Singh, E. Aikawa, Autophagy and its relevance in metabolic and degenerative disease. *Front. Endocrinol (Lausanne).* **3**, 266 (2020).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Ponnuswak, M. A. Mandell, T. Kimura, S. Chauhan, C. Cleyrat, V. Deretic, Secretory autophagy. *Curr. Opin. Cell Biol.* **35**, 106–115 (2016).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Ponnuswak, M. A. Mandell, T. Kimura, S. Chauhan, C. Cleyrat, V. Deretic, Secretory autophagy. *Curr. Opin. Cell Biol.* **35**, 106–115 (2016).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).

R. A. Nixon, The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).

M. Nollet, S. S.-Darmanin, V. Breuil, R. Al-Sahlian, C. Cros, M. Topi, D. Momin, M. Sanz, S. S. Sajdera, H. C. Anderson, Isolation and characterization of calcifying matrix vesicles in skeletal and soft tissue mineralisation. *Sci. Adv.* **8**, eabn1556 (2022).
Acknowledgments: We thank the Shaanxi Key Laboratory of Stomatology for technical support. Funding: This work was supported by the National Nature Science Foundation of China grants 81870787 (to K.J.), 82170978 (to K.J.), and 81870805 (to L.-n.N.); distinguished Young Scientists Funds of Shaanxi Province 2021JC-34 (to K.J.); and Shaanxi Key Scientific and Technological Innovation Team 2020TD-033 (to L.-n.N. and K.J.).

Author contributions: J.Y. and M.S. contributed equally to the experimental performing, data acquisition and analysis, and manuscript drafting. B.S. and D.C. contributed to data interpretation. W.L., Y.C., and X.H. contributed to animal experiments. Q.W., W.Q., and Z.Z. contributed to data analysis and interpretation. Y.L., S.Y., and J.K. contributed to electron microscopy experiments. K.J., L.-n.N., and F.R.T. contributed to the study conception and design, data interpretation, and manuscript revision. All authors have read and approved the current version of the manuscript.

Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 7 November 2021
Accepted 28 March 2022
Published 11 May 2022
10.1126/sciadv.abn1556