Calcified apoptotic bodies from PROCR+ fibroblasts initiate the tendon calcification at the early stages of heterotopic ossification

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Abstract

Heterotopic ossification (HO) comprises the abnormal formation of ectopic bone in extraskeletal soft tissue. The factors that initiate HO remain elusive. Herein, we found that calcified apoptotic bodies (CABs), which are secreted by PROCR$^+$ fibroblasts in the early stage of HO, lead to increased stiffness of the extracellular matrix. Specifically, single-cell transcriptome analyses of different stages of HO revealed a PROCR$^+$ fibroblast population that released CABs in the early stage of HO. CAB aggregation produced calcified nodules with high concentrations of calcium and phosphate, similar to those in calcified tendons. Annexin channels mediate calcium influx into CABs, which absorb to collagen I via electrostatic interaction. Functional inhibition of CABs significantly decreased the early stage microcalcification and inhibited HO of Achilles tendons. Thus, we revealed a pathological mechanism of HO initiation and identified CABs from PROCR$^+$ fibroblasts as the initiating factor of local microcalcification, creating an osteogenic microenvironment for HO.

Introduction

Heterotopic ossification (HO) comprises the abnormal formation of ectopic bone in extraskeletal soft tissue$^1$, which causes significant pain, swelling, joint stiffness and gradual limitation of movement, finally leading to disability$^2$. Current treatment strategies for HO comprise nonsteroidal anti-inflammatory drugs, bisphosphonates and surgical resection in severe cases$^3,4$. These treatments, however, have a low cure rate and a high recurrence rate, resulting in a large financial burden for patients$^5$. The bottleneck lies in our limited understanding of the cytological basis and microenvironmental changes in HO, which are determined by the abnormal cell fate of resident cells and the detrimental microenvironment, including blood vessels, nerves, immune cells, fibroblasts and tenocytes$^6$. Importantly, HO progression depends on the physical and chemical properties of the tendon microenvironment, including calcium and phosphorus deposition. Animal experiments have also shown that tendon stiffness increases significantly with the extension of healing time after tendon rupture$^7,8$.

Our previous studies suggested that osteoarthritis (OA) is initiated by pathological calcification and microcalcification expedites inflammation and accelerates disease progression$^9,10$. This explains why calcification progresses rapidly once microcalcification exists. Extracellular vesicles (EVs) are a heterogeneous population enveloped by a plasma membrane that have been implicated in the bone formation and mineral apposition, termed calcified EVs$^{11-15}$. We demonstrated that autophagy-derived microtubule-associated proteins 1A/1B light chain 3B (LC3)-positive calcified EVs from autophagosomes initiate pathological cartilage calcification in OA$^{16}$. In atherosclerosis (AS), calcified EVs released from macrophages and smooth muscle cells (SMCs) have been involved in the vascular calcification$^{17,18}$. These EVs provide a nurturing microenvironment for calcium phosphate nucleation and subsequent crystal growth within atherosclerotic plaques$^{19,20}$. The formation of microcalcifications causes plaque instability and correlates with cardiovascular risk$^{21}$. Although calcified EVs have been studied for their role in pathological calcification, their roles in HO are unknown.
Herein, we performed single cell sequencing of tendon cells from different stages of HO combined with scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryogenic-electron microscopy (cryo-EM), and atomic force microscopy (AFM) to clarify the stage-specific and location-specific pathology of minerals in HO tendons. Based on this model, we identified calcified apoptotic bodies (CABs) in HO tendon and investigated their origin and contribution to pathological calcification. CABs initiate pathological calcification in HO and the microcalcification-induced increased stiffness of the extracellular matrix (ECM) promotes the formation of a local osteogenic microenvironment. Our results revealed a new pathomechanism of HO initiation and identified CABs as a potential treatment target for HO.

**Results**

**Achilles tendon microcalcification leads to increased stiffness of the tendon ECM in the early stage of HO.**

The pathogenesis of HO involves initial changes in various cellular components combined with increased stiffness of the ECM, followed by progression of tendon osteogenesis and remodeling. Current treatment primarily focuses on the progression stages, while the initiation trigger is unknown. Therefore, we systematically investigated HO at different stages, especially initiation, using the rat Achilles tendon calcification model. Micro-computed tomography (CT) showed no radiographic evidence of bone formation at 1 week after Achilles tenotomy. Some ectopic bone nodules were identified in the Achilles tendons by 3 weeks. By 6 weeks, new bone (radio-opaque zones) in the Achilles tendon region was detected in 60% of tendons and in 100% by 9 weeks (Fig. 1a). Bone mineral density (BMD), bone volume/tissue volume (BV/TV), and bone surface/bone volume (BS/BV) analyses identified increased bone mass at different times in the HO group (Fig. 1b-d). Next, we identified tendon mineral distribution, morphology and composition at different HO stages. Macroscopically, alizarin red S staining indicated the presence of minerals at 1 week in HO group, which were increased at 3 weeks (Fig. 1e, 1f). TEM and SEM showed thickened and calcified collagen fibrils and microcalcifications that were widely dispersed in the tendon ECM the 1 week HO group. At 3 weeks, collagen banding disappeared and abundant minerals were found on the surface of collagen fibrils (Fig. 1e). These results indicated that HO development involves disordered tendon calcification. The chemical composition of these minerals was analyzed using energy-dispersive X-ray spectroscopy. There were significant variations in Ca and P contents in the 1 and 3 week HO samples (Fig. 1g, S1). The minerals in the samples were also characterized using cryo-EM. Selected area electron diffraction showed minerals along the collagen fibrils as early as 1 week. More extensive mineralization was identified after 3 weeks (Fig. 1e). New bone formation and mineralization were indicated using calcein at different time points (Fig. 1h). In the HO group at 3 weeks, the relatively fluorescence intensity of the calcein-labeled area (green; mineralization) was significantly higher than that at 1 week, both of which were higher than that in the sham group (Fig. 1i, S2). Micro-infrared analysis comparisons of the changes in collagen and the calcium phosphate content of Achilles tendon tissues in the sham and HO groups showed that in the HO group, the collagen content in the Achilles tendon
decreased and the phosphate content increased. Compared with that at 1 week after injury, the phosphate content at 3 weeks was significantly upregulated (Figure S3).

To correlate the nanomechanical profiles with the pathohistological results in the sham and HO groups, AFM analyses of in vivo tendon tissues were performed. We also assigned individual stiffness characteristics to specific tissue morphologies (stained with hematoxylin & eosin (H&E)) to perform more detailed measurements within defined regions of the samples (Fig. 1j), particularly in the early injury group. A map of stiffness values from the sham sample revealed an average stiffness of 2.63 N/m. In comparison, the microcalcified tendon from the 1 week HO group had an increased average stiffness of 2.99 N/m. The average stiffness of individual microcalcifications embedded in the ECM were further increased (3.47 N/m) and post-AFM H&E staining confirmed inflammatory cell infiltration of the injury site without the formation of osteoid (Fig. 1j, middle). The stiffness of a different areas of a representative sample from the 3 week HO group varied from 2.31 N/m to 4.03 N/m, indicating marked mechanical heterogeneity across the sample. Post-AFM H&E staining confirmed the extensive mineralization of the ECM in the 3 week HO group (Fig. 1j, bottom right, 1k). The correlation of local AFM data with matching histology corroborated that the stiffness peak represents typical microcalcification surrounded by softer ECM (Fig. 1j, 1l, S4). These results indicated that microcalcification formation was accompanied by increased matrix stiffness, especially in the early stage of HO. However, the cytological behavior underlying microcalcification requires study.

Single-cell RNA-sequencing (scRNA-seq) analysis identifies novel apoptotic-preferential PROCR+ fibroblasts in the early stage of HO.

Fibroblasts are the principal cell type in ligament tissue and the core player in the initiation of tissue ossification. However, the role and the underlying mechanism of fibroblast-mediated HO initiation and progression are unclear; therefore, we systematically investigated the dynamic cellular changes in fibroblasts from different stages of HO using 10x Genomics scRNA-seq. After unsupervised graph clustering of the 3 datasets combined (sham, 1 week and 3 weeks), the Seurat 3 RPackage was used to segregate the captured cells into 6 distinct cell clusters, including 2 principal cell types according to the cell assemblies on the t-distributed stochastic neighbor embedding (t-SNE) plot: the fibroblast cluster and the macrophage cluster (Fig. 2a, 2b). To annotate these populations, we plotted the fraction of cells expressing each marker across all stages using the differentially expressed genes (DEGs) among the cell types (from the heatmap in Figure S5). We investigated how the fractions of different cell clusters changed in the early stage of Achilles tendon injury. The number of fibroblasts decreased significantly after Achilles tendon injury after 1 week and 3 weeks (Fig. 2c, 2d). To further investigate the mechanism underlying cellular activity related to the formation of early microcalcifications at the single-cell level, we focused on fibroblasts. A t-SNE plot from the scRNA-seq analysis displayed 5 distinct fibroblast subpopulations with different gene expression patterns (Fig. 2e). Extra-skeletal mineralization is a consequence of apoptotic cell death or at least intersects with certain apoptotic signaling pathways and apoptosis is therefore a key factor in calcification. Notably, apoptosis score analyses identified Cluster 1 fibroblasts as the apoptotic-preferential cell population (Fig. 2f). The protein C receptor (PROCR)
was especially active in the apoptosis-related clusters, and immunostaining and flow cytometric analysis of tendon samples confirmed the presence of PROCR fibroblast subpopulations (Fig. 2g-2i). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses also revealed that PROCR fibroblast subpopulations was mainly enriched in functions such as cell-substrate adhesion, connective tissue development and cell migration (Fig. 2j, 2k, S6 and S7). These data revealed that the tendon fibroblasts comprised heterogeneous subpopulations and PROCR fibroblast were apoptotic preferential subpopulations in the early stage of HO.

**Calcified apoptotic bodies (CABs) from PROCR fibroblasts markedly aggregated and induced tendon microcalcification in the early stage of HO.**

Apoptotic bodies, which might directly contribute to pathological calcification, are released during cell apoptosis. Previously, we identified that calcified EVs initiate pathological calcification in OA; however, whether apoptotic PROCR fibroblasts release apoptotic bodies and directly induce HO initiation is unknown. H&E staining showed shrinkage of the nucleus and condensation and fragmentation of the chromatin in Achilles tendon fibroblasts in the HO group (Fig. 3a). Caspases execute apoptosis by selectively targeting and cleaving many key molecules in the cells. In the HO group, the percentage of cleaved caspase-3-positive and Tunel-positive fibroblasts was significantly increased during the inflammation phase at 1 week and decreased at 3 weeks (Fig. 3b-3e). Immunofluorescence staining for PROCR, also showed that the PROCR fibroblasts of 1 week HO group had abundant cleaved caspase-3 (Fig. 3f). Therefore, PROCR fibroblasts underwent apoptosis after Achilles tendon injury.

To further explore the pathomechanism of HO microcalcification, ultrastructure analysis of sham and HO tendons was performed. Magnified TEM images of 1 week HO tendons showed that the cell content was converted into many apoptotic bodies in the ECM via apoptosis (Fig. 3g, white arrows). Elemental analysis indicated that the apoptotic bodies contained both calcium and phosphate, and were thus termed calcified apoptotic bodies (CABs) (Figure S8). In the 3 week HO tendon, nuclei was fragmented into many discrete chromatin bodies because of nuclear DNA degradation. Each nuclear fragment was surrounded by a blebbled plasma membrane and these units (CABs) budded-off from the apoptotic cell. SEM images also demonstrated that the CABs aggregated into larger calcified nodules and were distributed along the collagen fibrils in the 1 and 3 week HO groups. No CABs were observed in any tendon samples from the sham group (Fig. 3h). In the 1-week HO group, elemental mapping analysis also confirmed that the CABs released from the fibroblasts, or around the disorganized collagen matrix, contained calcium, phosphorus, and nitrogen (Fig. 3i). CABs were also identified using calcein and cleaved caspase-3 staining at different time points and the quantity of the CABs peaked at 1 week after injury (Fig. 4a-4c). Thus, we confirmed the existence of CABs after Achilles tendon injury.

Next, we isolated the apoptotic bodies from tendons of sham and HO rats using a gradient centrifugation protocol and characterized them (Fig. 4d). Apoptotic bodies were not detected in the sham group; however, the representative cup shape appearance of EVs was observed. Compared with the EVs derived
from the sham tendon, the CABs from the HO tendons were larger and had an electron-dense internal structure resulting from calcium phosphate nucleation (Fig. 4e-4i). Nanoparticle tracking analysis revealed that the diameter of the CABs was 718.33 nm at 1 week post-injury and 836.67 nm at 3 week post-injury (Fig. 4i). Collectively, these results indicated the potential effect of PROCR+ fibroblast-released CABs on early stage HO microcalcification.

**Local high calcium and phosphorus caused by the CABs further aggravated their secretion from fibroblasts.**

Apoptotic fibroblast secretion of CABs resulted in large deposits of calcium and phosphorus in the ECM, which directly increased the stiffness and calcification of the microenvironment (Fig. 3i). However, whether microenvironment deterioration affected the remaining fibroblasts and promoted HO progression required investigation. We found that fibroblasts exposed to calcium and phosphate underwent apoptosis (Fig. 5a). Similar to what occurred *in vivo* in HO tendons (Fig. 3g), small calcifications entrapped by the collagen matrix fused to form larger calcified nodules, and the CABs aggregated to produce spherical calcified structures (Fig. 5a). Flow cytometry validated this result and showed that the proportion of apoptotic cells among the fibroblasts reached the peak at 7 days (Fig. 5b, c). CABs were also identified by alizarin red S and cleaved caspase-3 co-staining at 7 days (Fig. 5d). These results confirmed that fibroblast-derived CABs are involved in calcification.

TEM examination of fibroblasts cultured for 7 days showed that the CABs in the calcified group contained amorphous calcium and phosphorus (Fig. 5e-5h). In the calcified group, we observed the release of CABs from fibroblasts, which aggregated to produce calcified nodules with increased ECM stiffness (Fig. 5i, 5j, S9 and S10). Elemental mapping confirmed mineral Ca, P and N within the CABs (Fig. 5k). These data suggested that the CAB-induced high calcium and phosphorus microenvironment in turn aggravated the secretion of CABs from fibroblasts, forming a positive feedback loop to promote the Achilles tendon calcification.

**CABs initiate calcification in vivo by enriching calcium via Annexin channels.**

To explore whether transplantation of CABs could directly initiate HO *in vivo*, the rat intratendon implantation model was used. CABs were isolated from fibroblasts and the characteristics and functions of the CABs were examined. The isolated CABs contained electron-dense materials containing calcium and phosphorus (Figure S11a, S11b). Flow cytometry analysis of phosphatidylserine (PtdSer), a specific marker of apoptotic bodies, showed that the percentage of the CABs was 97.61% among the total EVs (Figure S11c). The CABs had a similar size to those seen *in vivo* in the HO tendons (Figure S11d). Protein expression of the CAB-specific makers (caspase-3 and cleaved caspase-3) was also verified (Figure S11e, S11f). Thus, the CABs were effectively isolated. Collagen scaffolds were then immersed in the CABs (1 mg mL⁻¹ CABs, 3.5 mM calcium ions, 2.1 mM phosphate ions) and implanted into the intratendon pockets of rats. Similar sized collagen scaffolds that were immersed in the solution (3.5 mM calcium ions, 2.1 mM phosphate ions) were used as controls. After 1 week, Micro-CT and 3D reconstruction of the
dissected legs in the collagen + CABs group showed calcification of the collagen scaffolds, but not in the controls (Fig. 6a). Ectopic bone was formed in the collagen + CABs group after 3 weeks (Fig. 6a). H&E staining was used to observe the features of ectopic bone formation in the rat intratendon implantation model. No ectopic bone was observed in the control group up to 3 weeks. A developed cancellous bone at 3 weeks post-surgery was clearly identified in the collagen + CABs group, but not in the control group (Fig. 6a, S12). Micro-CT revealed that the BV/TV ratios and BMD in the collagen + CABs group were significantly higher than those in the control group (P < 0.05, Fig. 6b, 6c). TEM images of specimens harvested at 1 week after implantation showed intense calcification of the collagen scaffolds in the CAB group. Collectively, these data indicated that ectopic mineralization can be induced \textit{in vivo} using CABs.

To further explore the mechanism of CAB-induced microcalcification, 3D self-assembled collagen fibrils were used to create a mineralization model. The collagen fibrils were examined using TEM to monitor the evolution of CAB-mediated mineralization in their native state. Mineral depositions were seen along the collagen fibrils as early as 3 days after immersion in mineralization medium (Figure S13a). More extensive intrafibrillar mineralization was identified after 5 days (Figure S13a). This provided compelling evidence that the CABs were adsorbed on the collagen fibrils and facilitated amorphous calcium phosphate (ACP) infiltration into the collagen fibrils, resulting in rapid mineralization.

Confocal laser scanning microscopy (CLSM) showed that the CABs were adsorbed on the collagen fibrils (Figure S13b), suggesting that CAB-collagen interaction might exist during collagen mineralization. To investigate the interactions involved in CAB-collagen binding, we employed Molecular dynamic (MD) simulation to provide detailed microscopic modeling at the molecular scale. MD simulation confirmed that CABs bind to collagen fibrils (Figure S14a-S14f). Calculation of the binding energy between CABs and collagen attributed the energy to electrostatic interaction. The contact area between the CABs and collagen was 7.38 nm$^2$, and the average electrostatic interaction energy, van der Waals interaction energy and total interaction energy were $-3928.33$, $-184.93$ and $-4113.26$ kJ/mol, respectively. Thus, electrostatic interaction is the predominant contributor to the CAB-collagen interaction at the overlap zone, and Arg11 and Arg14 of the three collagen chains contribute greatly to binding, whereas Glu13 repels proximity to the phospholipid membrane (Fig. 14d-14f).

A novel model system of culturing fibroblasts with high calcium and phosphorus medium was established to investigate the calcium uptake and release during mineralization by CABs. Phosphatidylserine (PS, apoptotic marker) and Fluo-4 (calcium marker) were not detected in the normal control group. After 6 hours, PS and calcium were clearly detectable, and were significantly upregulated after 12 hours in the CAB group. CAB formation was also observed using immunofluorescence microscope and TEM, which identified four stages: PS ectropion, calcium influx, crystal nucleon formation, and membrane structure penetration (Fig. 6e-6g, S15). The high Ca concentration in the CABs triggers their precipitation. When the membrane structure of the CABs was ruptured, the microcalcification was released into the ECM (Fig. 6f, S16). The SEM results revealed that mineral deposition and hydroxyapatite crystallization evolve from intermediate amorphous or poorly crystalline phases inside the membrane structure to more crystalline apatite phases that penetrate the membrane (Figure S16, S17).
To explore the function of Ca enrichment in CABs, mass spectroscopy (MS) was used to identify CAB membrane proteins. The MS/MS spectrum showed a series of ions in the mass range of 100–1800 that were derived from fragmentation of the peptide backbone. Proteomic analysis demonstrated that the CABs contain several ion channels. Ca\(^{2+}\) influx into CABs occurs through Annexin channels (Figure S18, S19). Immunogold and immunofluorescence labeling of Annexins on CABs confirmed the presence of Annexin channels. Importantly, blocking the Annexin channels using LCKLSL effectively inhibited the affinity of CABs for Ca\(^{2+}\) and their mineralization (Fig. 6i and 6j). Therefore, CABs continuously enriched for calcium through Annexin channels, subsequently inducing microcalcification of the ECM.

**CAB-induced microcalcification in the early stage of HO promotes M2 macrophage polarization.**

Macrophage polarization promoted the progression of HO. Do the CABs released during HO initiation further affect macrophage polarization? Immunofluorescence staining and TEM showed that macrophages were located near the microcalcification of loose connective tissue at 1 week after Achilles tendon injury (Fig. 7a and 7b). In our single cell data, we used previous published macrophage marker genes to identify the macrophages. Analysis of monocyte/macrophage subpopulations identified 3 different clusters (Fig. 7c). Heatmap analysis of gene expression in macrophage subclusters and subsequent pseudotime analysis revealed that the M1 cluster, situated at the earliest pseudotime, had the strongest proinflammatory capacity and later polarized into the M2 cluster with anti-inflammatory lineage fates (Fig. 7d-7f, S20). GO analyses showed that M1 macrophages associated with mechanical induction and inflammation, while M2 macrophages were associated with neurogenesis, angiogenesis, and osteogenesis (Fig. 7g and 7h).

To determine the potential role of stiffness in macrophage regulation in HO formation in injured Achilles tendons, we fabricated two kinds of polydimethylsiloxane (PDMS) substrates with different Young’s moduli (stiff, 15:1, and soft, 45:1 (PDMS elastomer: curing agent)). We seeded bone marrow-derived macrophages (BMDMs) onto the PDMS substrates to explore changes in cell behavior (Figure S21). We simulated the inflammatory environment using inflammatory factors and detected M1 macrophage activation. Following lipopolysaccharide treatment, M1 phenotype macrophages were seeded onto the PDMS substrates with different stiffness. After cell attachment for 24 h, the macrophages showed a larger spreading area in the stiff group than in the soft group. In addition, the mRNA levels of *Inos* and *Cd206* (M1 and M2 macrophage markers, respectively) were also detected, indicating that macrophages were activated and polarized from the M1 to the M2 phenotype in the stiff group. Furthermore, we detected relatively strong expression of angiogenesis and neurogenesis-related genes in the stiff group (Figure S22). Thus, these results suggested that macrophages are a heterogeneous cluster with distinct pseudotime lineage fates, with either proinflammatory or anti-inflammatory phenotypes. CAB-induced microcalcification promoted the polarization of M1 to M2 macrophages. Therefore, CABs in the initiation stage and macrophages in the progression stage represent therapeutic targets for HO.
Inhibition of CAB release and macrophage depletion delay the initiation and progression of HO, respectively.

Next, we investigated whether inhibition of CAB release and macrophage depletion were potential therapeutic targets for HO. Caspases play an essential role in apoptosis; therefore, their inhibition with the caspase inhibitor z-VAD-fmk has been used to reduce apoptotic cell death. Micro-CT and H&E staining were used to examine Achilles tendons harvested from rats at 6 weeks after achillotenotomy. The formed ossifications were almost completely lamellar bone, and bone marrows and trabecular bones were also observed. In contrast, the z-VAD-fmk treated rats failed to induce ectopic mineralization after apoptosis inhibition and quantitative analysis of BV/TV also identified a significant difference (Fig. 8a and 8c). H&E staining showed that at 1 week post-surgery, apoptosis of fibroblasts was partially inhibited by Z-VAD within the Achilles tendon. In addition, the fibroblasts underwent necrosis after apoptosis inhibition (Fig. 8e). Cleaved caspase-3 was clearly detectable in normal saline (NS) group but was significantly downregulated in z-VAD-fmk group (Fig. 8e and 8g). To determine the distribution of CABs during HO formation in the z-VAD-fmk group, immunofluorescent staining of the sections of injured tendon tissues from HO rats was performed. At 1 week post-surgery, CABs were induced as expected in the NS group, whereas significantly decreased CAB levels were observed in the z-VAD-fmk group (Fig. 8i and 8j). AFM revealed partial inhibition of the microcalcification associated with increased stiffness by z-VAD-fmk (Fig. 8i and 8k). Z-VAD-fmk also inhibited the polarization of from M1 to M2 macrophages (Fig. 8l and 8m). These results suggested that apoptosis is required for microcalcification formation in the early stage of injury and the later stage of bone formation.

To verify the importance of macrophages to HO formation in injured Achilles tendons, rats that underwent Achilles tenotomy were injected intravenously with clodronate–liposomes to deplete macrophages, using phosphate-buffered saline (PBS) liposomes as controls. Micro-CT showed that the clodronate-liposomes significantly reduced the HO volume compared with that in the PBS-liposomes group at six weeks post-surgery (Fig. 8b). The BV/TV in the clodronate–liposomes group was markedly lower than that in the PBS-liposomes group during HO pathogenesis (Fig. 8b and 8d). Similarly, H&E staining showed a decreased in mature bone tissues in the clodronate–liposomes group compared with that in the PBS-liposomes group in the injured Achilles tendons (Fig. 8b). Fibroblast apoptosis and cleaved caspase-3 levels were unaffected in the clodronate–liposomes group, while M2 macrophages were significantly depleted (Fig. 8f, 8h, 8l and 8m). Collectively, these results suggested that inhibition of CAB release and macrophage depletion delayed the initiation and progression of HO, respectively.

Discussion

Herein, we identified that CABs, specifically secreted by PROCR⁺ fibroblasts at early stage of HO, lead to ECM microcalcification (Fig. 9). Previous studies demonstrated that HO goes through a cartilaginous phase following trauma to connective tissue. In the present study, we reported that in the early stage of HO, the CABs secreted from PROCR⁺ fibroblasts aggregated to produce calcifying nodules, which cause increased stiffness of the ECM. Meanwhile, we identified the caspase inhibitor z-VAD-fmk as an approach
to suppress the progression of HO. Our results propose a novel paradigm to understand the progression of the early stage of HO and provide a good target for early intervention in ectopic mineralization diseases.

There has been a long-standing view that apoptosis is a passive phenomenon that serves as a homeostatic mechanism that controls the cellular component. As studies have shown, apoptosis is involved in OA and AS, as well as many other diseases and complications. In both human and animal OA models, the chondrocytes showed a morphology of apoptosis and hydroxyapatite microcrystals were also detected on their surface. In many vascular and vascular-related diseases (e.g., AS and hypertension), apoptosis precedes VSMC calcification and inhibiting apoptosis also inhibits calcification. Although it is very hard to intervene in the progression of HO at an early stage, the identified population of apoptosis-associated fibroblasts (PROCR⁺) provides a good target for HO therapy. In AS, matrix vesicle-like structures have also been found in calcified arteries and Kockx et al. showed that the structures were derived from SMCs and contained BAX, a proapoptotic member of the Bcl-2 family, indicating that they were the remnants of apoptotic cells. SMCs also exhibited reduced viability with increased apoptosis and higher expression of calcification markers (e.g., TNAP or Runx2). Generally, apoptotic bodies are considered to modulate the biological functions of neighboring cells by releasing a variety of substances, including microRNAs, lipids and proteins, that initiate signal transduction. Herein, we demonstrated that CABs play an important role in the formation of microcalcification in the early stage of Achilles tendon injury by enriching calcium. Therefore, the identified CABs are of great significance for the prevention and treatment of HO.

Although the pathological changes to the microenvironment underlying HO are complex, our group has focused on the formation of microcalcification in early stage of injury and the stiffness of the microenvironment. Our results showed that CABs from fibroblasts induce the microcalcification of Achilles tendon in the early stage of injury. In VC, the basic component of the mineral is hydroxyapatite (HAp) and nanoHAp has been identified to stimulate osteogenic differentiation and accelerate the mineralization of SMCs. In our study, multiple micro-nano analytical technologies were applied to study the nanoscale architecture and composition of HO. The calcification process started with spherical mineral particle formation in the ECM of the tendon, followed by densely packed material transformation deep into the ECM tendon. Our results also confirmed that local high calcium and phosphorus initiate apoptosis and the formation of microcalcification. In the early stage of Achilles tendon injury, a large amount of local cell death occurs, releasing a large amount of calcium and phosphorus ions into the ECM, and our data also showed a large increase in calcium and phosphorus concentrations at the early stage of Achilles tendon injury, which was confirmed using in vitro experiments. Increased stiffness of the ECM caused by microcalcification promotes the formation of a local microenvironment favoring neurogenesis and angiogenesis, resulting the heterotopic calcification. Therefore, the abnormal outcome of cell fate and the consequent change to the microenvironment lead to the occurrence of HO.
Inflammation, stem cell recruitment, chondrogenic differentiation, and finally, ossification are the four sequential pathological processes involved in heterotopic endochondral ossification. When tissue is injured during HO, macrophages, neutrophils and lymphocytes are recruited to the site of injury. Macrophages can acquire different functional phenotypes and promote mesenchymal stem cell (MSC) osteogenic differentiation, chondrogenic differentiation and angiogenesis by expressing cytokines and other factors such as the transforming growth factor-β1 (TGF-β1), bone morphogenetic protein (BMP), oncostatin M (OSM), substance P (SP), neurotrophin-3 (NT-3) and vascular endothelial growth factor (VEGF). In a model of neurogenic HO mice induced by spinal cord injury, macrophage-mediated inflammation was found to trigger neurogenesis. Moreover, activation of macrophages in an inflammatory situation contributes to neurogenic HO formation by secreting OSM. Our study provided evidence that minerals formed on the tendon of HO induced the polarization of macrophages from pro-inflammatory (M1) to anti-inflammatory (M2) phenotypes, with gradually altered transcriptional profiles and functional outputs. Furthermore, we evaluated the potential role of stiffness in macrophage regulation in HO formation in injured Achilles tendons. Previous studies have focused more on the immune cells, such as monocytes and macrophages, and this is the first report of the involvement of microcalcification in the early stage of Achilles tendon injury. Although the injury-inflammation-stem cell recruitment-regeneration process is common in wound healing, importantly, we demonstrated that in the inflammation stage of HO, macrophages induce the formation of a local osteogenic microenvironment by sensing the formation of a hard matrix.

**Conclusion**

No strategies or drugs are available to prevent crystal deposition and permit mineral dissolution in HO tendons, emphasizing the importance of revealing the pathogenic mechanism underlying HO to identify therapeutic targets. This study showed that in the early stage of injury, microcalcification of the Achilles tendon occurred together with increased stiffness of extracellular matrix. Moreover, failure to regulate apoptosis can result in the damage to organs or organisms. Our results showed that CABs from PROCR+ fibroblasts induce microcalcification of the Achilles tendon in the early stage of injury. A better understanding of these phenomena could result in new strategies to control the calcification process by inhibiting the early stages of hydroxyapatite deposition induced by CABs.

**Materials and methods**

The group designation and experimental scheme are described in Figure S23. The detailed method for Micro-CT analyses, histologic and immunohistochemistry evaluation, TEM, SEM, elemental mapping, calcein fluorescent labeling and histomorphometrical analysis, Fourier transform infrared spectroscopy, single-cell RNA-sequencing (scRNA-seq) analysis, isolation of apoptotic bodies, nanoparticle-tracking analyses, western blotting, cell culture, PDMS substrates, live-cell imaging and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) are included in the supplementary information.
**Rat Achilles tenotomy model and treatment**

*In vivo* experiments were conducted with male Sprague-Dawley rats, aged 8 weeks, weighing 200–300 grams, obtained from the Fourth Military Medical University's Laboratory Animal Research Center. Fourth Military Medical University's Institutional Animal Care and Use Committee approved all surgical procedures for those experiments (ethics approval number: 20220906). After each rat was anesthetized, Achilles tendon was exposed by incising the skin and subcutaneous tissue. The Achilles tendon of HO group was transected along the midpoint, while the Achilles tendon of sham group was not transected. During the entire experimental period, none of the rats died and there was no difference between the right and left legs in the occurrence of ectopic bone formation.

**Atomic force microscopic**

AFM (Asylum Research, Santa Barbara, CA, USA) was used to analyze 15 μm sections of the tendon tissue from different groups. Micromorphological imaging of the tendon tissue was performed using a silicon probe (PPP-NCLR-20, Nanosensors, Neuchatel, Switzerland) with a force constant of 42 N/m and a resonance frequency of 161 kHz after the hydrated sections were naturally dried. All measurements were repeated for 3 positions of each tissue sample and the values were averaged.

**Cryogenic-electron microscopy (cryo-EM)**

Quantifoil Jena R2/2/gold grids coated with holey-carbon (2 μm hole size) supporting film (Electron Microscopy Sciences, Hatfield, PA, USA) were plasma-treated and used within 30 min after treatment. Sections (90 nm thick) were obtained and dropped on the surface of the grids. After vitrification (~1000 Å thick), the grids were transferred to a Gatan 626 cryo-transfer holder and maintained at a temperature below -170 °C during cryo-EM observation with a Talos F200C microscope (FEI, Hillsboro, OR, USA) at 100 kV. The electron dose for each exposure was 20e Å⁻². Selected area electron diffraction was performed to confirm the crystallinity of the minerals in tendon.

**Flow cytometry analysis**

For flow cytometry analysis of tendon cells, tendon tissues were harvested and minced with scissors, then enzymatically digested in CO₂-independent incubator shaker (Kuhner, ISF1-XC) with 1 mg/ml Collagenase I (Sigma-Aldrich, SCR103) and IV mixture (Sigma-Aldrich, C4-28-100MG) for 3 h at 37°C. After diluted with serum-free medium and centrifuged at 200 g for 10 min, the cell pellets were resuspended in ACK lysis buffer (ThermoFisher, NC9067514) to remove blood cells. Prior to staining, the cells suspended in FACS buffer comprised of PBS with 5% BSA (Sigma-Aldrich, SRE0096) were filtered through a 40 μm mesh. Dissociated single cells were stained with anti-PROCR (bs-9506R, Bioss, Beijing, China) and anti-rabbit IgG (H+L) Fluor647-conjugated secondary antibodies (SA00014-9, Proteintech, Wuhan, China). The detection was performed using a flow cytometer (CytoFLEX, Beckman Coulter, Brea, CA, USA). The flow cytometric analysis was performed with FlowJo 10.0 software (Flow Jo LLC, Ashland, OR, USA) after washing twice by centrifugation at 600 g for 5 min with ice-cold DPBS + 2% FBS.
Apoptotic bodies re-suspended in PBS were incubated with Annexin V-PE (Annexin V-PE Apoptosis Detection Kit, MedChemExpress, New Jersey, USA) and the Ca\(^{2+}\) marker (Fluo-4, AM ester, US Everbright, Suzhou, China) and mixed for 30 min. After washing the apoptotic bodies 3 times with 2% BSA in PBS, Annexin V and Ca\(^{2+}\) detection was performed using a flow cytometer (CytoFLEX, Beckman Coulter, Brea, CA, USA). Apoptosis in fibroblasts was assessed using Annexin V Apoptosis detection kit (Annexin V-PE, Propidium Iodide (PI) solution and Annexin V binding buffer). FACS analysis of the fibroblasts that are in early (annexin V+/PI\(\bar{2}\)) or late (annexin V+/PI\(+\)) apoptotic phase was performed using the FlowJo 10.0 software (Flow Jo LLC, Ashland).

3D self-assembled collagen fibrils

An acetic acid/collagen stock solution (5 mg/mL) derived from rat tail tendon was used for 3D self-assembled collagen fibrils. The self-assembled collagen solution was dropped on a 400 mesh Au TEM grid and dried at room temperature. The grids were rinsed with deionized water 3 times and air-dried. For the cell assay, room-temperature drying was also performed on the collagen solution.

In vivo pathological collagen mineralization models

The intratendon ectopic calcification model was created in rats (n = 3). The apoptotic bodies were seeded into 3D collagen scaffolds (ACE Surgical Supply Co., Brockton, MA, USA) under sterile conditions. The 3D collagen scaffolds (5 mm × 5 mm) were implanted intratendonly into separate pockets in 6-week-old rats. After 3 weeks, the rats were sacrificed and their Achilles tendons were collected for subsequent analysis.

Co-culture of the apoptotic bodies and collagen

The collagen solution was dropped on a 400-mesh nickel TEM grid and air-dried. The collagen-coated grids were floated upside down over solutions of apoptotic bodies (250 \(\mu\)g/mL) from different groups and imaged using TEM.

Mass Spectrometry Analysis of apoptotic bodies.

After isolation, the apoptotic bodies were treated with Radioimmunoprecipitation assay lysis buffer. Then, the mixture was injected into the mass spectrometer (Orbitrap Eclipse Tribrid Mass Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) after trypsin digestion. After calibrating using the standard compounds, the mass spectrometer was operated in the data-dependent mode. In this mode, the mass spectrometer cycled between full MS scans with m/z 100–1800. Only proteins with high protein false discovery rate confidence were considered for further analysis.

Calcium channel blockers

We employed the small Annexin A2 inhibitor molecule, LCKLSL hydrochloride (MedChemExpress), to study the function of Annexins.
**Molecular dynamic simulation**

Molecular dynamic simulations were conducted between DPPC (Di Palmitoyl Phosphatidyl Choline)/DPPS (Di Palmitoyl Phosphatidyl Serine) bilayers (DPPC:DPPS = 1:1) with the triple-helix region of collagen type I (PDB ID: 7CWK) using the GROMACS 2021.5 package. Lipid and protein were parameterized by Lipid21 and Amberff14sb force field. An initial structure with a 12 × 12 × 15 nm³ cubic box of lipid bilayer containing 234 DPPC and 234 DPPS was constructed at the CHARMM-GUI website and collagen was 1.4 nm away from the bilayer, then system was dissolved in TIP3P water (transferable intermolecular potential with 3 points; 48717 water molecules) and 231 Na⁺ were added to maintain electrical neutrality. Energy minimization was performed using the steepest descent algorithm with a force tolerance of 500 kJ mol⁻¹ nm⁻¹. Periodic boundary conditions were imposed in all 3 directions. Then, these systems were relaxed for 1 ns under NPT MD simulations, and position restraints with a constant of 1000 kJ mol⁻¹ nm⁻² in three directions were performed on heavy atoms of lipids and proteins.

Next, 200 ns NPT MD simulations were performed on the bilayer-collagen system. Pressure was maintained at 1 bar using a Parrinello-Rahman barostat in an semiisotropic manner (xy and z directions) and the temperature was maintained at 310 K using a V-rescal thermostat. The LINCS algorithm was performed for the constrained bond lengths of hydrogen atoms. Lennard-Jones interactions were calculated within a cutoff of 1.2 nm, and electrostatic interactions beyond 1.2 nm were treated using the particle-mesh Ewald (PME) method with a grid spacing of 0.16 nm. UCSF ChimeraX was used to visualize the results.

**Z-VAD-FMK Injections.**

To inhibit fibroblast apoptosis in the Achilles tendon, Z-VAD-FMK (178603-78-6, Sigma-Aldrich, St. Louis, MO, USA) dissolved in normal saline was injected into rats at 1 μg/rat, 3 times a week. An equal volume of normal saline was injected into the sham group.

**Macrophages depletion**

For macrophage depletion, the injured rats were injected intravenously with clodronate-liposomes (5 μL/g) immediately after Achilles tenotomy and every 3 days for 6 weeks post-surgery. The control group received an equivalent volume of PBS-loaded liposomes under the same conditions.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± standard deviation. In order to test the normality and homoscedasticity assumptions of the corresponding data sets, the Shapiro-Wilk test and modified Leven test were used. The differences between groups were evaluated using the Student's t test, one-factor or two-factor analysis of variance (ANOVA) followed by Holm-Šidák multiple comparison tests. To ensure
the validity of the observations, quantitative experiments were repeated at least 3 times. For all tests, statistical significance was preset at $\alpha = 0.05$.

**Declarations**

**Data availability**

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Information.

**Code availability**

No custom computer code or custom algorithm was used in this study.

**Competing interests statement**

The authors declare no conflict of interest.

**References**


47. Huang, Y. et al. Macrophages in heterotopic ossification: from mechanisms to therapy. NPJ Regenerative medicine 6, 70 (2021).


Figures
Figure 1

Microcalcification of the Achilles tendon leads to increased stiffness of the tendon ECM in the early stage of HO. a Micro-computed tomography (micro-CT) images of Achilles tendons of rats from the sham and HO groups after 1, 3, 6 and 9 weeks. Heterotopic ossification was observed in the HO group (white arrows in a). Scale bar, 5 mm. b-d Quantitative analysis of bone histomorphometric parameters based on the micro-CT images in a: (b) bone mineral density (BMD), (c) bone volume/tissue volume (BV/TV) and (d)
bone surface/bone volume (BS/BV). e Alizarin red S staining of Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks. Areas stained with alizarin red S indicate calcified regions. Alizarin red S, red; DAPI, blue. Scale bar: 100 μm. Representative TEM, SEM, and Cryo-EM images of the Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks. f Quantitative analysis of the calcified regions stained with Alizarin red S in (e). g Elemental analysis of the tendon minerals in the 1 week HO group. h Fluorescent labeling observations of calcium using calcein. i Quantitative analysis of the calcified regions in (h). j Post-AFM histological overview and representative AFM stiffness map of the Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks. Young's modulus increased after tendon injury compared with that in the sham group. k Quantitative analysis of osteoid volume based on H&E staining images in j. l Quantitative analysis of AFM stiffness based on j. Data represent the means ± standard deviations (n = 3). Statistical analyses were performed using one-way ANOVA with a post-hoc Tukey's test. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 2

Single-cell RNA-sequencing (scRNA-seq) analysis identifies novel apoptotic-preferential PROCR⁺ fibroblasts in the early stage of HO. a The workflow depicting the collection and processing of specimens of sham and HO tendons for scRNA-seq. b Dimension reduction presentation (via tSNE) of combined single-cell transcriptome data from Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks (n = 14402). Each dot represents a single cell and is labeled with corresponding cell categories and
is colored according to its cell type identity. Clusters were generated using a resolution of 0.2 before subclustering into major cell types according to the Methods. The Seurat 3 R-Package segregation grouped the cells into 6 distinct cell clusters. **c, d** Quantitative analysis of clusters based on the combined single-cell transcriptome data in b. **e** tSNE of fibroblast clusters (FB1–FB5). **f** Apoptosis score analyses of fibroblasts based on e. **g** Bioinformatic analysis of PROCR\(^+\) cell populations based on e. **h** Immunofluorescence microscopy of Achilles tendons of rats from the sham group. PROCR, green; PDGF receptor-\(\alpha\), red; DAPI, blue. Scale bar: 10 \(\mu\)m. **i** FACS gating strategy of PROCR\(^+\) cells from Achilles tendons of the sham group. **j** Representation analysis of GO categories showing different functions for PROCR\(^+\) cells. **k** Representation analysis of KEGG categories showing different functions for PROCR\(^+\) cells.
Figure 3

The calcified apoptotic bodies (CABs) from PROCR+ fibroblast markedly aggregated and induced tendon microcalcification in the early stage of HO. a H&E staining of Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks. Scale bar: 50 μm. High magnification of the black rectangle outline in the low magnification image showing apoptotic cells (arrows). Scale bar: 10 μm. b Representative images of immunohistochemical staining of Achilles tendons derived from the sham and HO groups after 1 and 3 weeks.
weeks. Scale bar, 50 μm. c Representative images of TUNEL staining from the sham and HO groups after 1 and 3 weeks. Scale bar, 50 μm. d Quantitative analysis of cleaved caspase-3+ cells in b (n = 3). e Quantitative analysis of TUNEL+ cells in c (n = 3). f Representative images of immunofluorescence staining of an Achilles tendon derived from the HO group after 1 week. PROCR, red; Cleaved caspase-3, green; DAPI, blue. Scale bar, 10 μm. High magnification of the white rectangle outline in the low magnification image showing the apoptotic PROCR+ fibroblasts (arrows). Scale bar: 5 μm. g Representative TEM images of Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks. Scale bar: 1 μm. High magnification of the red rectangle outline in the low magnification image showing the cells and apoptotic bodies (arrows). Scale bar: 300 nm. h Representative SEM images of Achilles tendons of rats from sham and HO groups after 1 and 3 weeks. Scale bar: 1 μm. High magnification of the red rectangle outline in the low magnification image showing apoptotic cells (arrows). Scale bar: 500 nm. i SEM images and elemental mapping showing the aggregation of the CABs in the 1-week HO group. Scale bar: 20 μm. Data represent the means ± standard deviations (n = 3). Statistical analyses were performed using one-way ANOVA with a post-hoc Tukey’s test. **P < 0.01, ***P < 0.001.
Figure 4

**Characterization and isolation of the CABs in HO.**

- **a** Immunofluorescence microscopy of Achilles tendons from the sham and HO groups after 1 and 3 weeks. Ca (*in vivo*), green; Cleaved caspase-3, red; DAPI, blue. Scale bar: 50 μm.
- **b** Quantitative analysis of the colocalization of the free green dots and red dots of Achilles tendons from sham and HO groups after 1 and 3 weeks (n = 3).
- **c** Quantitative analysis of the relative fluorescence intensity of cleaved caspase-3 (n = 3).
- **d** Schematic representation of the isolation of CABs.

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**a**

<table>
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<th>Sham</th>
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<th>3 weeks</th>
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<td><img src="merge_3_weeks.png" alt="Image" /></td>
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**b**

![Bar chart comparing the percentage of colocalization between sham and HO groups.](chart_colocalization.png)

**c**

![Bar chart comparing the relative fluorescence intensity between sham and HO groups.](chart_intensity.png)

**d**

1. **Sham** → **Tissue slicing** → **Enzymatic hydrolysis** → **Gradient centrifugation** → **ABs**
2. **HO** → **Tendon** → **IVs** → **Centrifugation** → **ABs**

---

**Figure 4**

**Characterization and isolation of the CABs in HO.**

- **a** Immunofluorescence microscopy of Achilles tendons from the sham and HO groups after 1 and 3 weeks. Ca (*in vivo*), green; Cleaved caspase-3, red; DAPI, blue. Scale bar: 50 μm.
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- **c** Quantitative analysis of the relative fluorescence intensity of cleaved caspase-3 (n = 3).
- **d** Schematic representation of the isolation of CABs.
apoptotic bodies. e Flow cytometry of Annexin V and Ca$^{2+}$ on apoptotic bodies of Achilles tendons from the sham and HO groups after 1 and 3 weeks. f TEM images of the apoptotic bodies of Achilles tendons from the sham and HO groups after 1 and 3 weeks. Scale bar: 1 μm. High magnification of the red rectangle outline in the low magnification image showing the apoptotic bodies (arrows). Scale bar: 200 nm. g, h The corresponding quantification of percentage of apoptotic bodies of Achilles tendons from sham and HO groups after 1 and 3 weeks (n = 3). i Nanoparticle-tracking analyses (NTA) of apoptotic bodies of Achilles tendons of rats from the sham and HO groups after 1 and 3 weeks (n = 3). Data are presented as the means ± standard deviations (n = 3). Statistical analyses were performed using one-way ANOVA with a post-hoc Tukey’s test. ns, no significance. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5

Local high calcium and phosphorus caused by the CABs further aggravated its secretion from the fibroblasts. a Phase contrast microscope images of the fibroblasts cultured in the control and calcified medium for 3, 5, 7 and 10 days. Scale bar, 100 μm. b Flow cytometry of Annexin V and PI of the fibroblasts cultured in the control and calcified medium for 5, 7 and 10 days. c The corresponding quantification of the percentages of apoptotic fibroblasts cultured in the control and calcified medium for
Representative images of immunofluorescence staining of fibroblasts cultured in the control and calcified medium for 7 days. Alizarin red S, red; Cleaved caspase-3, green; DAPI, blue. Scale bar, 10 μm. High magnification of the white rectangle outline in the low magnification image showing the CABs (arrows). Scale bar: 5 μm. 

Representative TEM images of fibroblasts cultured in the control medium for 7 days. Scale bar: 1 μm. Representative TEM images of fibroblasts cultured in the calcified medium for 7 days. Scale bar: 1 μm. High magnification of the red rectangle outline in the low magnification image showing the CABs (arrows). Scale bar: 500 nm. 

Electron diffraction of the mineral precursors within the CABs. 

Elemental analysis of the regions depicted by the arrows in f. 

Representative SEM images of fibroblasts cultured in the control medium for 7 days. Scale bar: 5 μm. Representative SEM images of fibroblasts cultured in the calcified medium for 7 days. Scale bar: 3 μm. High magnification of the white rectangle outline in the low magnification image showing the CABs. Scale bar: 500 nm (left), 200 nm (right). 

SEM images and elemental mapping showing the aggregation of CABs in the calcified group. Scale bar: 20 μm. Data are presented as the means ± standard deviations (n = 3). Statistical analyses were performed using one-way ANOVA with a post-hoc Tukey’s test. ***, P < 0.001.
Figure 6

**CABs initiate calcification in vivo by enriching calcium through Annexin channels.**

- **a** Micro-computed tomography (micro-CT) and H&E staining images of the rat intratendon implantation model from the collagen and collagen + CABs groups after 1 and 3 weeks. Scale bar, 5 mm (left); 100 μm (right).
- **b, c** Quantitative analysis of bone histomorphometric parameters based on the micro-CT images in a: (b) bone mineral density (BMD) and (c) bone volume/tissue volume (BV/TV).
- **d** TEM image of the mineral
deposition within the calcified plaque in the collagen + CABs group (red rectangle). Scale bar: 1 μm. e Immunofluorescence microscopy of fibroblasts cultured in the calcified medium for 24 h and the CABs (indicated with white circle). Phosphatidylserine, red; Fluo-4, green; DAPI, blue. Scale bar, 500 nm. f Representative TEM image of CABs indicated with a red circle. Scale bar, 500 nm. g Schematic representation of CAB-mediated enrichment of calcium at the four stages referred to in e. h Immunogold and immunofluorescence labeling of Annexins on CABs (arrows) in the vicinity of the ECM calcification in the calcified group. Scale bar: 500 nm (left); 1 μm (right). i Immunofluorescence microscopy and TEM images of CABs incubated with collagen I for 3 days. Scale bar: 5 μm (left); 200 nm (right). j Quantitative analysis of the relative intensity of the fluorescence of calcium based on i (n = 3). Data are presented as the means ± standard deviations (n = 3). Statistical analyses were performed using one-way and two-way ANOVA with post-hoc Tukey’s tests. ns, not significant. **P < 0.01.
Figure 7

The microcalcification induced by the CABs in the early stage of HO promotes M2 macrophage polarization. 

- **a** Representative confocal images of Achilles tendons stained with F4-80 (red), calcein (green) and DAPI (blue) in rat tendons at 1 week after surgery. Scale bars, 50 μm.

- **b** Representative TEM images of Achilles tendons at 1 week after surgery. Scale bars, 1 μm.

- **c** tSNE of macrophage clusters (M0–M2 macrophage). Clusters were generated using a resolution of 0.2 before subclustering into major...
cell types according to the Methods. \textit{d-f} Pseudotime analysis of macrophage clusters (M0–M2 macrophage). \textit{g} Representative analysis of GO categories showing the different functions of M1 macrophage. \textit{h} Representative analysis of GO categories showing the different functions of M2 macrophage.

\textbf{Figure 8}
Inhibition of CAB release and macrophage depletion delay the initiation and progression of HO, respectively. a, b Micro-CT images (scale bars, 5 mm) and H&E staining (scale bars, 100 μm) of heterotopic calcification in the Achilles tendons 6 weeks after surgery from the NS, z-VAD-fmk, PBS-lip and Clo-lip groups. c, d Quantification of the BV/TV based on a, b. e, f Representative images of H&E staining and immunohistochemical staining of the Achilles tendons derived from the NS, z-VAD-fmk, PBS-lip and Clo-lip groups. Scale bar, 50 μm. g, h Quantitative analysis of cleaved caspase-3⁺ cells in e, f (n = 3). i Immunofluorescence microscopy and representative AFM stiffness map of Achilles tendons of rats from the NS and z-VAD-fmk groups. Calcein, green; Cleaved caspase-3, red; DAPI, blue. Scale bar: 10 μm. j Quantitative analysis of the colocalization of the free green dots and red dots of Achilles tendons of rats (n = 3). k Quantitative analysis of AFM stiffness based on i (n = 3). l Representative confocal images of M2 macrophages (CD206 positive) of Achilles tendons of rats from the NS, z-VAD-fmk, PBS-lip and Clo-lip groups. Scale bars, 50 μm. m Quantitative analysis of macrophage phenotype from the NS, z-VAD-fmk, PBS-lip and Clo-lip groups. Data are presented as the means ± standard deviations (n = 3). Statistical analyses were performed using one-way ANOVA with a post-hoc Tukey’s test. ns, no significance. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 9**

<table>
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<tr>
<th>Fibroblast apoptosis</th>
<th>Calcified apoptotic bodies</th>
<th>Matrix hardness</th>
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<td>PROCR⁺ fibroblasts release the calcified apoptotic bodies.</td>
<td>The calcified apoptotic bodies induce the microcalcification in ECM.</td>
<td>The calcified collagens promote the formation of a local microenvironment for osteogenesis.</td>
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The CABs secreted by PROCR$^+$ fibroblasts at early stage of HO leads to the microcalcification in ECM. PROCR$^+$ fibroblasts release CABs in the early stage of HO and the CABs aggregate to produce calcifying nodules in the ECM. The CABs are continuously enriched with calcium via Annexin channels and induce ECM microcalcification. Microcalcification induced by the CABs increases the stiffness of the ECM, creating an osteogenic microenvironment for HO.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- EditorialPolicyChecklist.pdf
- ReportingSummary.pdf
- Supplementarymaterials.docx
- NCOMMS2323657RS.pdf