Vascular Smooth Muscle Cell Calcification Is Mediated by Regulated Exosome Secretion

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Rationale: **Matrix vesicles (MVs), secreted by vascular smooth muscle cells (VSMCs), form the first nidus for mineralization and fetuin-A, a potent circulating inhibitor of calcification, is specifically loaded into MVs. However, the processes of fetuin-A intracellular trafficking and MV biogenesis are poorly understood.**

Objective: **The objective of this study is to investigate the regulation, and role, of MV biogenesis in VSMC calcification.**

Methods and Results: **Alexa488-labeled fetuin-A was internalized by human VSMCs, trafficked via the endosomal system, and exocytosed from multivesicular bodies via exosome release. VSMC-derived exosomes were enriched with the tetraspanins CD9, CD63, and CD81, and their release was regulated by sphingomyelin phosphodiesterase 3. Comparative proteomics showed that VSMC-derived exosomes were compositionally similar to exosomes from other cell sources but also shared components with osteoblast-derived MVs including calcium-binding and extracellular matrix proteins. Elevated extracellular calcium was found to induce sphingomyelin phosphodiesterase 3 expression and the secretion of calcifying exosomes from VSMCs in vitro, and chemical inhibition of sphingomyelin phosphodiesterase 3 prevented VSMC calcification. In vivo, multivesicular bodies containing exosomes were observed in vessels from chronic kidney disease patients on dialysis, and CD63 was found to colocalize with calcification. Importantly, factors such as tumor necrosis factor-α and platelet derived growth factor-BB were also found to increase exosome production, leading to increased calcification of VSMCs in response to calcifying conditions.**

Conclusions: **This study identifies MVs as exosomes and shows that factors that can increase exosome release can promote vascular calcification in response to environmental calcium stress. Modulation of the exosome release pathway may be as a novel therapeutic target for prevention. (*Circ Res*. 2015;116:1312-1323. DOI: 10.1161/CIRCRESAHA.116.305012.)**

Key Words: extracellular matrix ■ exosomes ■ vascular calcification

Vascular calcification is the accumulation of calcium phosphate salts in the medial and intimal layers of the vessel wall and is a common complication in patients with chronic kidney disease, diabetes mellitus, and atherosclerosis.[1] The earliest phase of mineralization is thought to occur via a process similar to that observed during bone formation, where chondrocytes and osteoblasts, in response to physiological signals, secrete small, specialized membrane-bound bodies termed matrix vesicles (MVs) which act to nucleate calcium phosphate (Ca/P) crystals in the form of hydroxyapatite.[2–4]

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In the vessel wall, in response to pathological signals such as inflammatory cytokines or a mineral imbalance, vascular smooth muscle cells (VSMCs) undergo osteo/chondrogenic conversion. This is characterized by expression of bone-related proteins and the release of MVs; however, the origin and mechanisms leading to release of these particles is poorly understood.[4,5] Electron microscopy (EM) studies have shown that vesicles form the first nidus for mineralization and localize in close proximity to elastin and collagen fibrils.[3,4,6,7] They

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Nonstandard Abbreviations and Acronyms	
Ca/P	calcium and phosphate
CRMV	collagenase-released matrix vesicle
ECM	extracellular matrix
EM	electron microscopy
GO	gene ontology
MV	matrix vesicle
MVB	multivesicular body
NTA	nanoparticle tracking analysis
SMPD3	sphingomyelin phosphodiesterase 3
VSMC	vascular smooth muscle cell

are heterogeneous in size and mineral content and appear to originate from both apoptotic and living VSMCs at sites of medial calcification, as well as from VSMCs, macrophages, endothelial cells, and platelets in atherosclerotic plaques.[2,8–10]

Importantly, healthy VSMCs efficiently prevent vascular calcification by expression of calcification inhibitors, some of which are loaded into MVs.[2] In addition, they take up from the circulation the potent calcification inhibitor fetuin-A, a glycoprotein secreted predominantly by the liver.[11,12] Fetuin-A seems to be recycled by VSMCs and loaded into MVs where it can act to bind mineral and stabilize it against further growth.[13] Circulating fetuin-A levels are reduced in patients with calcification[14] while in vitro, prolonged exposure of VSMCs to procalcific factors such as elevated Ca/P, in the absence of fetuin-A, causes MVs to become mineralization competent due, in part, to exposure of phosphatidyl serine and annexin A6 nucleation sites on the vesicle membrane.[6,12]

Despite the clinical importance of MVs in triggering vascular calcification, little is known of the mechanisms of MV biogenesis and fetuin-A loading. Definitive studies on the regulation of MV release and their subcellular origins have not been performed, but it is thought that they are formed by budding from the plasma membrane.[2] However, recently fetuin-A was found in urinary exosomes, which originate from intracellular multivesicular bodies (MVB).[15] These data challenge our current views on the plasma membrane origin of MVs and suggest that the exosomal pathway may be implicated in biomineralization.

Methods

An expanded Materials and Methods is available in the Online Data Supplement.

Cell Culture and Transfection

Human aortic VSMCs were isolated from medial explants and cultured as described previously.[2] Calcifying conditions were the addition of increased extracellular calcium (2.7–5.4 mmol/L) and phosphate (2.5 mmol/L) as indicated.

Fetuin-A Labeling, Uptake, and Live-Cell Exocytosis

Bovine fetuin-A (Sigma) was labeled using an Alexa488 labeling kit in accordance with the manufacturer's protocol (Invitrogen). For uptake experiments, VSMCs were serum-starved for 16 hours and then incubated with Alexa488-labeled fetuin-A (10 μg/mL) for 30 to 180 minutes at 37°C.

For live-cell tracking and immunofluorescence, VSMC MVBs were labeled with 5 μmol/L N-rhodamine–labeled phosphatidylethanolamine (Avanti Polar Lipids) as described previously.[16] VSMCs were incubated with 20 μg/mL Alexa488-labeled fetuin-A for 1h at 37°C. Time-lapse acquisitions of optically sectioned z-volumes were captured using Leica TCS SP5 confocal microscope (Leica Microsystems).

Isolation of Vesicles

MVs and apoptotic bodies were isolated by differential ultracentrifugation from VSMC culture medium and cell lysates prepared as previously described.[2,6] In some experiments, 2.5 μmol/L GW4869 or 1 μmol/L Y27632 was added to the cell culture media.

Nanoparticle Tracking Analysis

VSMC-derived MV nanoparticle tracking analysis (NTA) was performed using the light scattering mode of the NanoSight LM10 (NanoSight Ltd, Amesbury, United Kingdom).

Quantitative Reverse Transcriptase Polymerase Chain Reaction, Immunoblotting Analysis, and Densitometry

VSMC RNA was reverse transcribed using Mu-MLV reverse transcriptase and the SYBR quantitative polymerase chain reaction assay (Eurogentec) according to manufacturer's protocol. Immunoblotting and densitometry were as previously described.[6]

Flow Cytometry

Flow cytometry analysis of VSMCs and aldehyde/sulfate latex bead coupled MVs was conducted as described[6] using BD FACScalibur (BD Bioscience).

Quantification of exosomes secreted in the cell culture media was as previously described with modifications.[17] Arbitrary units were calculated as mean fluorescence units×percentage of positive beads and normalized to the number of viable VSMCs.

Tissue Analysis

Normal and calcified human vessel samples were obtained from surgeries with appropriate ethical approval, and immunohistochemical staining was performed as described previously.[4] Transmission EM was performed on human vessel rings obtained from patients with chronic kidney disease as previously described.[4]

Proteomic Analysis of VSMC MVs

VSMC MVs (40 μg) were separated in a Tris-glycine 5% to 20% polyacrylamide gradient gel and subjected to mass spectrometry/mass spectrometry analysis (LTQ-Orbitrap XL).

Similarity between diverse protein sets was estimated using the Jaccard coefficient defined as the number of common proteins between 2 sets divided by the total number of proteins in the 2 sets. The Jaccard coefficient range is 0 to 1 where 1 indicates that 2 sets are identical.

Statistical Analysis

Data shown are mean±SD. All data were verified in ≥3 independent experiments. Statistical analysis was performed by 1-way ANOVA with Bonferroni post hoc test or Student *t* test as appropriate using PRISM software (GraphPad). Values of $P<0.05$ were considered statistically significant.

Results

Fetuin-A Is Recycled by VSMCs via Late Endosomal/MVB Compartments

Alexa488-labeled fetuin-A was rapidly internalized by VSMCs and appeared within the cytoplasm in small punctate structures where it colocalized with the early endosome marker, EEA-1 (early endsome antigen 1; Figure 1A). Uptake was the same in the presence or absence of unlabeled protein (Online Figure IA), suggesting receptor-independent, liquid

flow type internalization of fetuin-A. This mechanism was supported by data showing that dynosore, an inhibitor of the small GTPase dynamin, did not affect fetuin-A uptake, whereas uptake of a clathrin-dependent protein, transferrin, was inhibited by 50% (Online Figure IB) and no colocalization of internalized fetuin-A with clathrin was observed (Online Figure IC). Additionally, small interfering RNA knockdown of the putative fetuin-A endocytic receptors, annexin A2 and annexin A6,[18] had no effect on fetuin-A delivery to the early endosomes (Online Figure IIA–IIC).

After 3 hours, fetuin-A accumulated in numerous intracellular vesicles in the perimembranous and juxtanuclear regions of the cell (Figure 1B and 1C), where it colocalized with CD63 (Figure 1B), LAMP-1 (lysosomal associated membrane protein; Figure 1C), and LAMP-2 (data not shown), suggesting that fetuin-A is targeted to the late endosomal compartment and lysosomes.

A specific subset of the late endosomal compartment, namely MVBs, are involved in the exosome secretion pathway[19] and fetuin-A accumulated within rhodamine-labeled MVBs (Figure 1D). Moreover, time-lapse confocal microscopy showed intracellular double-positive vesicles dynamically disappeared from the VSMCs indicating exocytosis of fetuin-A via the MVB pathway (Online Movies I and II).

We also tracked Alexa488-labeled fetuin-A in a pulse-chase experiment and showed that incubation of VSMCs for 4 hours in fetuin-A free media resulted in virtually complete disappearance of internalized fetuin-A (Online Figure IIIA). Treatment with the lysosomal inhibitor bafilomycin A1, before withdrawal of fetuin-A from the media, completely restored the intracellular distribution of fetuin-A (Online Figure IIIA), indicating that some fetuin-A is targeted for lysosomal degradation.

VSMC MVs Are Exosomal-Like Vesicles

The above data suggest that MVB-localized fetuin-A is recycled via the exosomal pathway for subsequent release from the cell. EM preparations of VSMC-derived MVs support this notion as they are small, 100-nm diameter, membrane-bound vesicles similar in size and morphology to previously described exosomes[20] (Figure 2A) and are enriched with the exosomal tetraspanins CD9 and CD63 compared with apoptotic bodies and VSMC lysates on Western blot (Figure 2B). CD9 and CD63 were also abundant on the plasma membrane and surface of MVs as revealed by flow cytometry (Figure 2C), but MVs displayed weak expression of the plasma membrane protein, CD71, and were enriched with MHC I (major Histocompatibility complex)[21] consistent with an exosomal origin (Figure 2B and 2C).

Lysosomal membrane proteins have previously been detected in exosomes[22] and LAMP-1 and LAMP-2 were present on VSMC-derived MVs (Figure 2B and 2C); however, they lacked the lysosomal enzyme, cathepsin D, suggesting that lysosomes are not involved in MV biogenesis (Figure 2D). Similarly, endoplasmic reticulum resident proteins, calnexin and protein disulfide-isomerase, were not enriched in VSMC MVs (Figure 2D), but acetylcholinesterase activity was detected (Figure 2E), consistent with other exosome studies.[23] Exosomes also contain specific cytoplasmic and endosomal proteins[20] and VSMC MVs were positive for a subset of cytosolic proteins including α smooth muscle actin and vinculin, as well as the exosomal protein, Tsg101[20] (Figure 2D).

Exosome formation in MVBs is triggered by hydrolysis of sphingolipids and release of ceramide in a reaction catalyzed by sphingomyelin phosphodiesterase 3 (SMPD3; also known as neutral sphingomyelinase 2).[24] SMPD3 synthetic

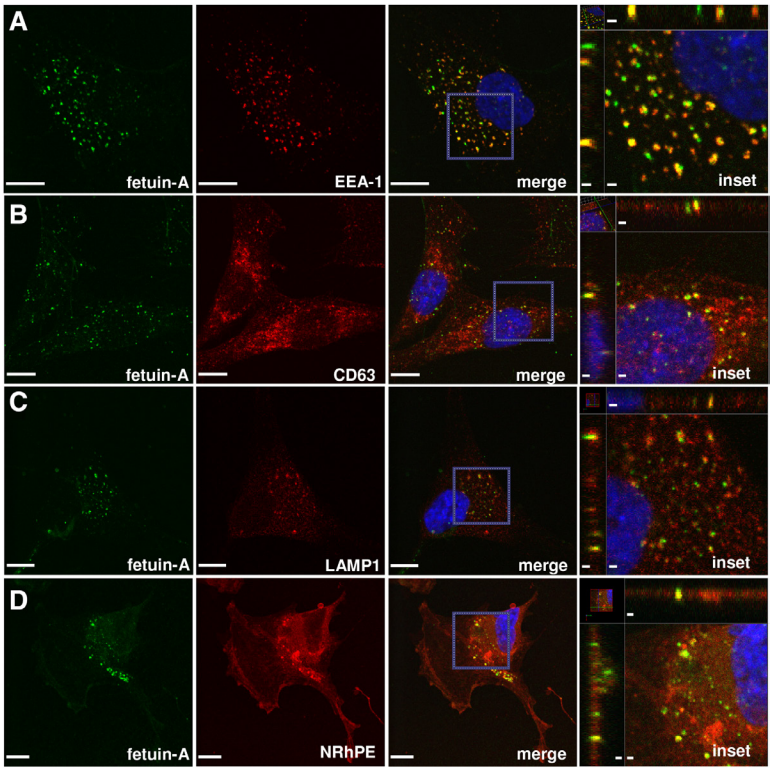

Figure 1. Internalization and intracellular distribution of fetuin-A. A, Alexa488-labeled fetuin-A is taken up by human vascular smooth muscle cells and within 30 minutes appears in early endosomes (EEA-1 [early endsome antigen 1]) (**B**–**D**). In 3 hours, fetuin-A is detected in late endosomes and lysosomes (CD63 and LAMP-1 [lysosomal associated membrane protein]) and colocalizes with N-rhodamine-labeled phosphatidylethanolamine (N-Rh-PE), marking multivesicular bodies. Scale, 10 μm. Boxes highlight the inset region Scale, 1 μm.

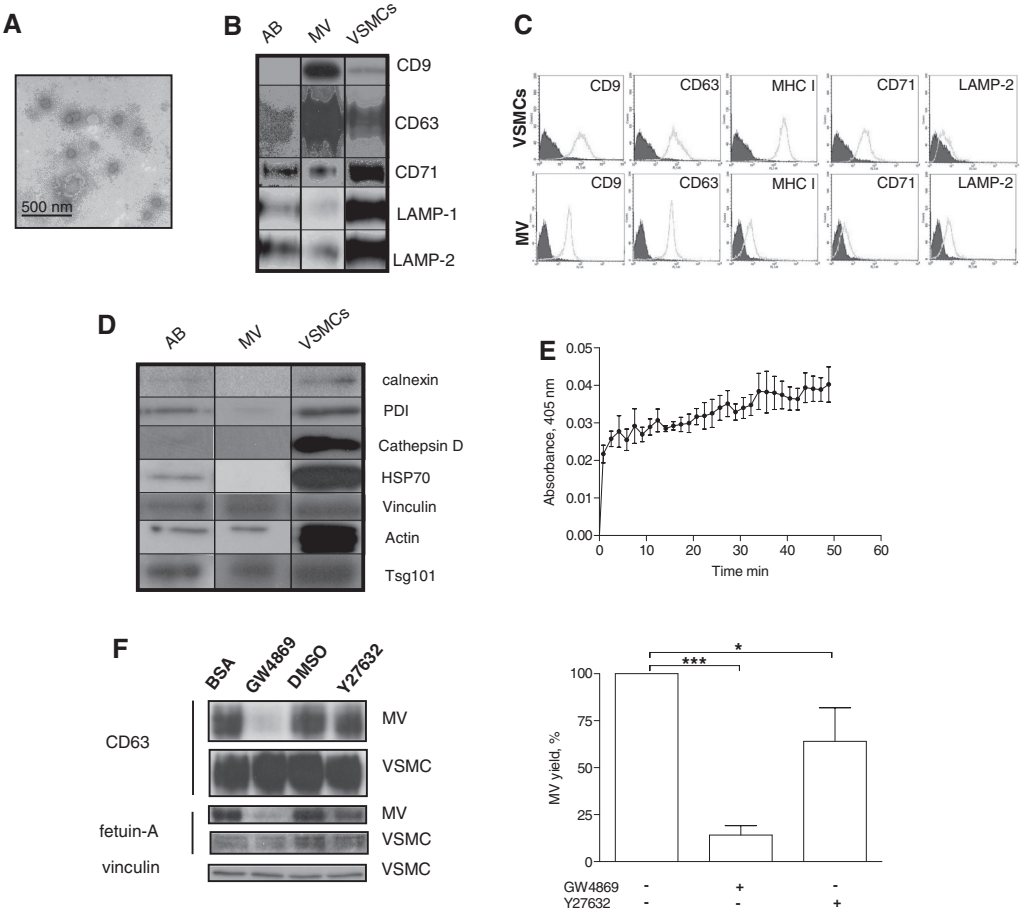

Figure 2. Vascular smooth muscle cell (VSMC)–derived matrix vesicles (MVs) are exosomes. A, Electron microscopy of VSMC exosomes/MVs. Scale, 500 nm. **B**, Western Blot shows enrichment of exosomal markers, CD9 and CD63 in VSMC-derived MVs, compared with VSMC and AB (apoptotic bodies) lysates, but not the plasma membrane marker, CD71. **C**, Flow cytometry analysis of MVs and nonpermeabilized VSMCs showing enrichment of exosomal (CD9, CD63) and endolysosomal (LAMP-1 [lysosomal associated membrane protein]) markers on MVs compared with enrichment for plasma membrane markers (CD71 and MHC I [major Histocompatibility complex]) on the VSMC surface. **D**, Western blot shows lack of endoplasmic reticulum (calnexin and PDI) and lysosomal (cathepsin D) markers in MVs. Cytosolic proteins (HSP70 [heat shock protein 70], vinculin, and α-SM actin) were selectively present in MVs and the endosome-specific protein (Tsg101). **E**, Acetylcholinesterase activity was detected in MVs (mean±SD, N=4). **F**, **left**, Treatment with the SMPD3 inhibitor 2.5 μmol/L GW4869 for 16 hours blocked MV release. Note loss of CD63 and fetuin-A. **Right**, Quantification by densitometry (mean±SD, N=3), **P*<0.05; ****P*<0.001, ANOVA.

inhibitors efficiently abrogate exosome release and VSMCs treated with GW4869 showed dramatically reduced exosome release (less than ≈14.1±5% of control values), and this was accompanied by a decrease in the amount of fetuin-A and CD63 recovered in MV pellets (Figure 2F). In addition, inhibition of exosome release with GW4869 induced accumulation of fetuin-A in VSMCs (Online Figure IIIB). In contrast, an inhibitor of plasma membrane blebbing, Y-27632 which selectively inhibits Rho-associated coiled coil forming protein serine/threonine kinase,[25] decreased the production of MVs down to 64±17.8% but had only a moderate effect on fetuin-A secretion (Figure 2F), suggesting that fetuin-A recycling and VSMC exosome secretion are regulated by SMPD3.

Proteomic Analysis of VSMC-Derived Exosomes

To elucidate specific structural and functional features of VSMC-derived exosomes, we analyzed their protein composition (Online Figure IV and Online Table I). Using mass spectrometry, we identified 345 proteins (full list see Online Table I). Protein enrichment by cellular component analysis identified that enriched proteins originated from different cellular compartments including the plasma membrane and cytosol (Figure 3A and Online Table II) while biological processes analysis revealed that the most abundant proteins were implicated in cell adhesion (14.7%, gene ontology [GO]:0007155), cell motion (13.3%, GO:0006928), and regulation of cell death (12.7%, GO:0010941; Figure 3A; Online Table II). Protein enrichment based on molecular functions identified the majority of VSMC exosomal proteins as involved in nucleotide (20.7%, GO:0000166) and calcium ion binding (15.8%, GO:0005509; Figure 3A; Online Table III), which are both key to the regulation of mineralization.[26,27] Using available online databases, we also performed a comparison with specialized vesicles isolated from the extracellular matrix (ECM) of mineralizing osteoblasts (collagenase-released MV [CRMV]) by collagenase treatment and secreted MVs isolated from mineralizing osteoblast medium (medium MV).[28] Both showed a similar functional enrichment profile with VSMC-derived exosomes including nucleotide

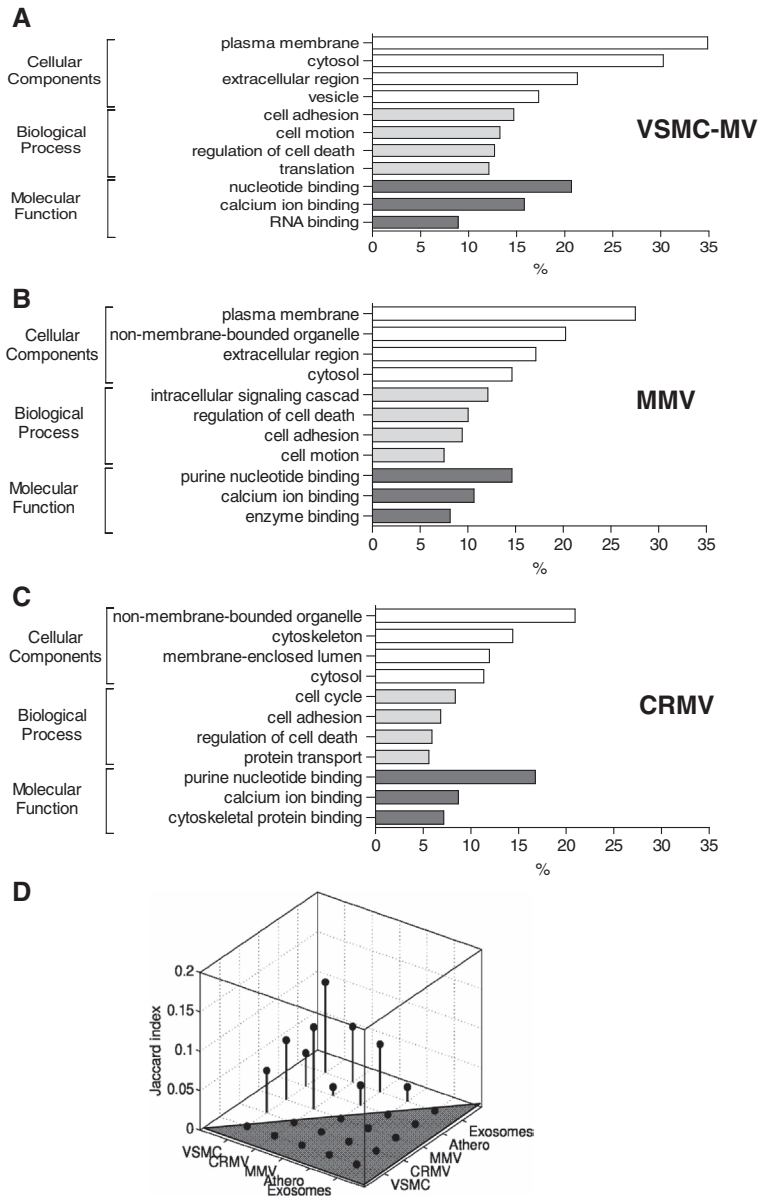

Figure 3. Proteomic analysis of vascular smooth muscle cell (VSMC)–derived exosomes. **A**, Bar plot showing percent enrichment of VSMC-derived exosomes, (**B**) osteoblast-derived matrix vesicles (MMVs), and (**C**) osteoblast extracellular matrix-derived MVs (CRMVs) and Gene Ontology terms. **D**, Stem plot demonstrating Jaccard similarity coefficients for proteomes of VSMC-derived exosomes (VSMC), MVs derived from osteoblast media (MMV) and osteoblast extracellular matrix (CRMV), microparticles isolated from atherosclerotic lesions (athero), and the online exosome protein database (exosomes). 0 indicates no similarity and 1 indicates complete similarity. The self-comparison data sets are not shown.

and calcium-binding proteins (Figures 3A–3C; Online Tables IV–VIII). However, while the vesicles isolated from the media of both VSMCs and osteoblasts (medium MVs) were enriched with plasma membrane proteins, osteoblast matrix bound vesicles (CRMVs) were enriched with organelle and cytoskeletal proteins, suggesting that they may be of different origin.

Further comparative analysis of overlapping proteins between VSMC-derived exosomes and CRMVs revealed 78 common proteins including ECM proteins previously implicated in bone development and calcification (Online Table IV). The first group include proteins that directly bind calcium and form either nucleation sites (annexins A1, A5, and A6) or can accelerate mineral growth (collagens type I, V, VI, and XII).[27] The second group contains multifunctional ECM proteins regulating cell survival and differentiation such as fibulin-1, as well as integrin-binding proteins osteonectin/SPARC (secreted protein acidic and rich in cysteine), periostin, and transforming growth factor-β–induced, which are all important regulators of bone development.[29] Several heparan sulfate proteoglycans (perlecan/heparan sulfate proteoglycan 2 and versican) are also involved in ECM organization and modulate growth factor signaling, thus affecting osteogenesis and bone formation.[30] In addition, 2 proteins (reversion-inducing cysteine-rich protein with kazal motifs [RECK] and a disintegrin and metalloproteinase [ADAM9]) are directly involved in ECM proteolytic remodeling, which precedes mineralization.[31]

Next, we compared the proteomic content of VSMC-derived exosomes with microvesicles obtained from atherosclerotic lesions,[9] the online exosome protein database,[32] as well as the CRMV and medium MVs datasets.[28] The Jaccard index of similarity (the ratio of the number of common proteins to the total number of proteins in 2 groups of interest; Figure 3D) revealed that the highest proportion of VSMC exosome proteins were present in the exosome (exosomes) and MVs derived from osteoblast media (medium MV) datasets, whereas the

similarity with microvesicles obtained from atherosclerotic lesions and osteoblast-derived matrix MVs (CRMV) was lower (Figure 3D). The full list of overlapping proteins is presented in Online Table IV.

Taken together, these results suggest that VSMC-derived exosomes are derived from the same cellular compartment as a subset of osteoblast MVs and are enriched with proteins implicated in bone development and calcification. However, their proteomic composition and functional signature also implicate them in cell motion and adhesion and closely resembles that of exosomes secreted by other nonmineralizing cell types. Their reduced similarity with vesicles from atherosclerotic plaques is consistent with the multiple cellular origins of vesicles in this tissue where VSMC exosomes only represent a portion of the total population.[8–10]

Extracellular Calcium Enhances Exosome Release and Triggers Calcification

Next, we investigated whether exosome secretion by VSMCs is modulated by factors that promote calcification and whether exosomes were crucial for calcification. NTA revealed that VSMCs release a population of vesicles ranging in size from 30 to 520 nm with an average mode size of 147±5.9 nm (n=5; Figure 4A; Online Video III). Exosome secretion was increased nearly 2-fold in calcifying conditions induced by elevated calcium and phosphate (control 4.92±0.46 E8 particles/mL and calcifying 9.89±0.26 E8 particles/mL), but there were no effects on their size distribution (mode size 136±3.6 nm), suggesting a similar origin for vesicles released in both conditions (Figure 4A). To rule out the possibility that the particles detected by NTA were not other mineral containing particles such as calciprotein particles or inorganic Ca/P crystals formed by precipitation in the calcifying media, we also analyzed the signature of these particles.[33] Both showed a different size distribution to that of exosomes excluding their contribution (Online Figure V).

To quantify further exosomes, we immobilized CD63 antibodies on beads to capture exosomes from the media, which were then detected by flow cytometry by staining with fluorescently labeled CD81 antibodies (Online Figure VIA and VIB). Control experiments showed that this assay could not detect Ca/P precipitates (Online Figure VIC). Knockdown of SMPD3 using Small interfering RNA reduced exosome secretion (Online Figure VID), and consistent with the NTA, treatment of VSMCs in calcifying conditions resulted in a significant increase in exosome secretion (Figure 4B), which was associated with upregulation of SMPD3 mRNA expression (Figure 4C).

To examine the role of exosomes in VSMC calcification, we inhibited exosome release using the chemical inhibitors, spiroepoxide and 3-O-Methyl-Sphingomyelin. Notably, both inhibitors reduced VSMC exosome production (Figure 4D) and prevented VSMC calcification (Figure 4E and 4 F; Online Figure VIE). Conversely, the addition of exosomes to calcifying VSMCs markedly enhanced mineralization (Figure 4G).

VSMC-Derived Calcifying Exosomes Contain a Mineral Phase

Using transmission EM, a subpopulation of exosomes released by VSMCs, only under calcifying conditions, contained a dark precipitate composed of Ca/P as detected by EDX analysis (energy dispersive X-ray; Figure 5A; Online Figure VIIA and VIIB). The mineral was a noncrystalline Ca/P salt, as a diffraction pattern was not observed (data not shown). Importantly, the rounded appearance of the calcified exosomes was significantly different from the needle-like crystalline Ca/P salts indicative of in vitro generated calciprotein particles or Ca/P precipitates isolated from calcifying media, and these particles were never observed in EM analyses of VSMC exosomal pellets (Figure 5B; Online Figure VIIC and VIID).

To confirm that the mineralized rounded structures observed on EM were indeed CD63-positive exosomes, we incubated exosomes isolated from VSMCs treated in normal or calcifying conditions, with CD63-capture beads (Online Figure VIC) and used a bisphosphonate labeled with Fluo (BP-Fluo) to detect mineral (Figure 5C; Online Figure VIIIA and VIIIB). We observed low but consistently increased binding of BP-Fluo to calcifying exosomes indicative of Ca/P salts (Figure 5D and 5E). Apoptotic bodies from calcifying VSMCs also bound significantly more BP-Fluo (Online Figure VIIIC).

Cytokines and Growth Factors Regulate Exosome Secretion by VSMCs

Next, we examined what other factors might influence exosome secretion. Transforming growth factor-β1 or low-serum conditions significantly reduced VSMC proliferation (Online Figure XE), increased expression of smooth muscle markers, and induced changes in cell morphology (Online Figures IXB, IXC, XB, and XIB) while addition of platelet derived growth factor-BB (Online Figure IXC) had the opposite effect, consistent with the induction of a synthetic phenotype (Online Figure XC and XF). Exosome quantification revealed that transforming growth factor-β1 decreased exosome production ≈4.3× while platelet derived growth factor-BB increased it ≈1.8× (Figure 6A and 6B). Treatment of VSMCs with platelet derived growth factor-BB increased calcification in calcifying conditions (Figure 6C; Online Figure XIC–XIF) while VSMCs in low serum showed abrogated calcification (Online Figure XIE), suggesting that increased exosome release promotes calcification. Cytokines also influenced exosome secretion including tumor necrosis factor-α which upregulated exosome secretion ≈1.5× (Figure 6D) while IL-6 and interleukin-10 both reduced exosome secretion (Online Figure XIA).

MVBs and Exosomes Are Detectable in Calcified Arteries

We quantified by EM MVBs in VSMCs in vessels obtained from controls and patients with chronic kidney disease on dialysis.[34] No MVB-like structures were observed in the healthy vasculature (Figure 7A and 7B) but were present in VSMCs from patients on dialysis. Treatment of vessel rings ex vivo for 5 days in calcifying conditions increased the number of MVB-like structures in both control and dialysis vessels (Figure 7A and 7B).

Immunohistochemical staining of arterial samples showed that the exosomal marker CD63 was not present in the normal vessel wall; however, extensive CD63 staining was observed in calcified arteries (Figure 7C; Online Table IX). In the arterial media, CD63 colocalized with α-smooth muscle actin–positive VSMCs in areas negative for CD68 macrophage staining (Figure 7C and 7D; Online Figure XII). This pattern

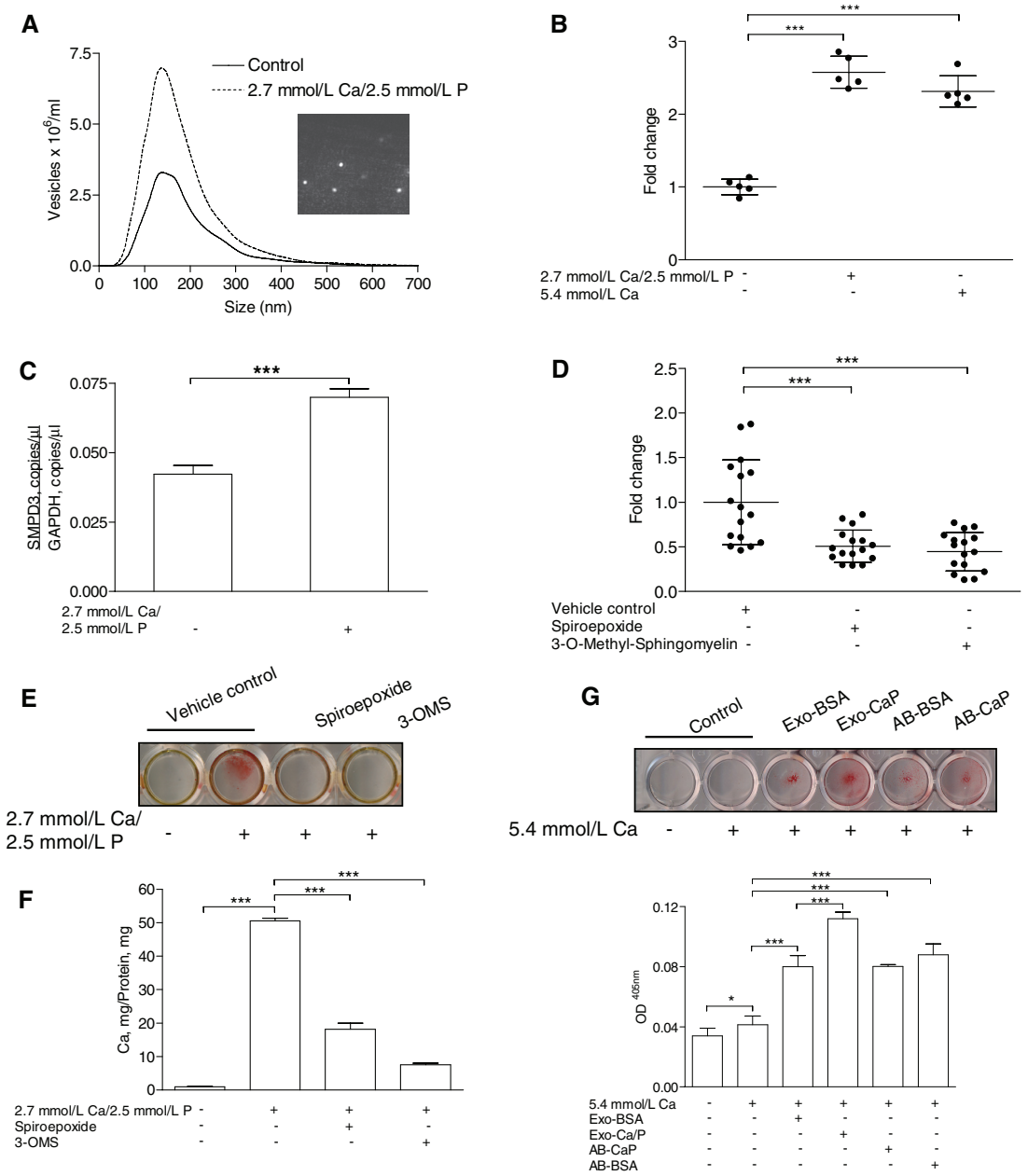

Figure 4. Sphingomyelin phosphodiesterase 3 (SMPD3) regulates exosome secretion and calcification. **A**, Nanoparticle tracking analysis of exosomes derived from vascular smooth muscle cells (VSMCs) in control and calcifying conditions for 16 hours showing size distribution and quantification. Inset shows exosomes (white spots) visualized with scattered light. **B**, Increased exosome secretion in response to calcium quantified using CD63-coated beads and fluorescence-activated cell sorter analysis. AU indicates arbitrary units (mean±SD, N=5). **C**, VSMCs treated in calcifying conditions for 16 hours showed increased SMPD3 expression by quantitative polymerase chain reaction. **D**, VSMCs treated in the presence of vehicle control (dimethyl sulfoxide, 0.1%) spiroepoxide (10 µmol/L), or 3-O-Methyl-Sphingomyelin (3-OMS, 16.5 µmol/L) showed reduced exosome secretion (mean±SD). Inhibition of SMPD3 reduced VSMC calcification as revealed by Alizarin Red staining (**E**) and o-Cresolphthalein Complexone assay (**F**). Addition of exogenous exosomes and ABs (1 µg) stimulated calcification in calcifying conditions. **G**, Alizarin Red was quantified after extraction (below).

overlapped with that of S100A4 a marker of synthetic VSMCs, as well as annexin A6, another abundant VSMC exosomal marker[6] while only minor or no staining was observed for Lamp-1 and Cathepsin D, markers that were not present in exosomes (Online Figure XII). Notably, in vessels from children on dialysis with increased calcium load, CD63 staining often preceded deposition of calcification detected with von Kossa staining (Figure 7C; Online Table IX), suggesting that deposition of exosomes is an early event in vascular calcification.

To test this further, we extracted vesicles, using collagenase digestion, from aortic samples showing early signs of calcified particle deposition[35] (Figure 7E). CD63-bead capture and CD81 detection on the surface of these vesicles confirmed the presence of intact exosomes (Figure 7F). CD9 and CD63 as well as fetuin-A and α-smooth muscle-actin were detected by Western blot (Figure 7G) while CD68 was not detected in the vesicle lysates, suggesting that the exosomes were VSMC derived.

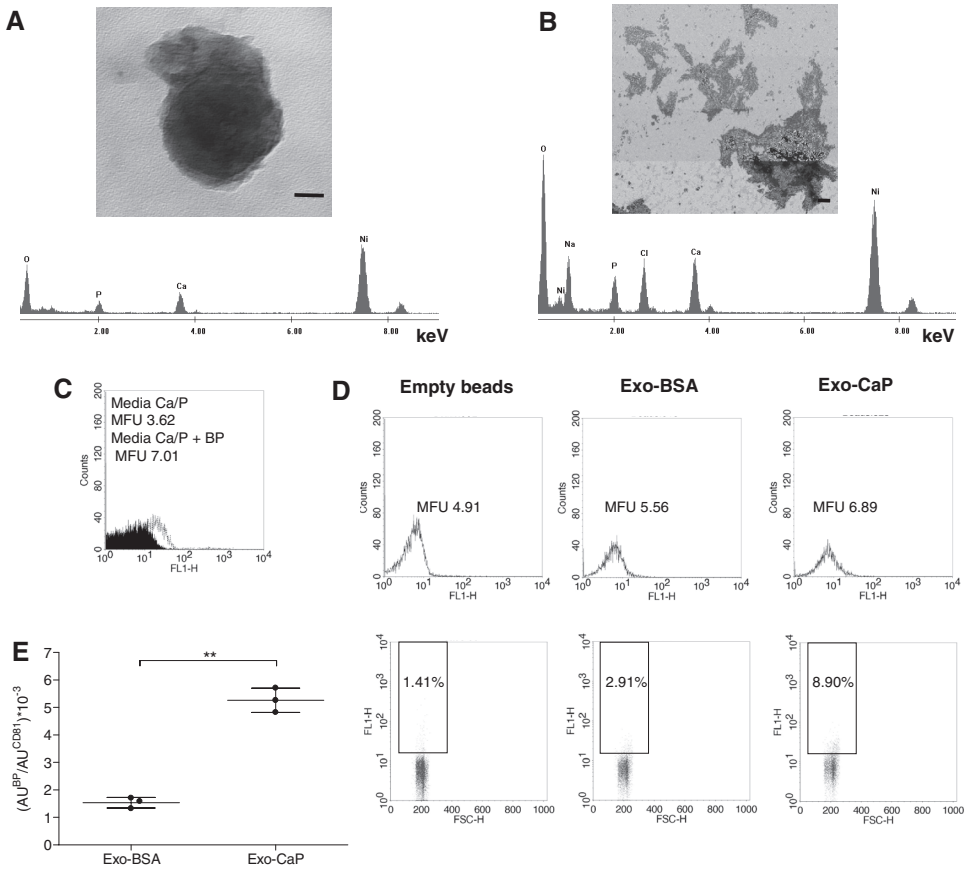

Figure 5. Exosomes contain calcium and phosphate (Ca/P). Electron microscopy/EDX (energy dispersive X-ray) analysis of exosomes isolated from calcifying vascular smooth muscle cells (VSMCs), Size bar, 20 nm (**A**) and Ca/P crystals formed in the absence of cells, Size bar, 100 nm (**B**). Note peaks for calcium and phosphate, carbon has been removed from the graphs. **C**, Detection of Ca/P with BP-Fluo using flow cytometry. **D**, Detection of Ca/P in exosomes isolated from control and calcifying VSMCs (2.7 mmol/L Ca+2.5 mmol/L P) (Exo-BSA and Exo-CaP) using CD63 bad capture and BP-Fluo (5 μg/mL) detection by flow cytometry. **E**, Quantification of flow cytometry data by normalization to CD81-PE antibody (mean±SD, N=3). Representative data from 3 independent experiments (**P<0.01).

Discussion

In this study, we describe for the first time the origin of VSMC-derived MVs and identify them as exosomes emanating from intracellular MVBs. We show that exosome release correlates with the ability of VSMCs to calcify and show they are secreted in response to upregulation of SMPD3, with their release dynamically regulated by osteogenic stimuli as well as cytokines and growth factors. Importantly, we show for the first time that exosomes can calcify and are enriched in the calcified vasculature. Proteomics revealed that VSMC exosomes shared similarities with osteoblast-derived MVs but were most similar to exosomes from nonmineralizing cell types, suggesting that they are likely to participate in processes beyond calcification. Exosome release was increased in proliferating VSMCs and exosomes were deposited in precalcified vessels, suggesting that increased exosome release, at sites of vascular injury, may prime the vessel wall to calcify. Ultimately, exosome mineralization is initiated when calcification inhibitors are absent or dysfunctional and extracellular calcium is elevated (Figure 8).

Fetuin-A Is Recycled by VSMCs via an Exosomal Pathway

Previously, VSMC-derived vesicles were defined as MVs by their functional similarity to similar membrane vesicles involved in bone mineralization, but their exact origin remained poorly characterized.[2,36] However, uptake and trafficking of fluorescently labeled fetuin-A identified VSMC MVs as exosomes, formed by inverted budding into MVBs, and released by fusion of the MVB membrane with the plasma membrane.[19,20] VSMC-derived vesicles were enriched with exosomal markers such as CD63, and their production and fetuin-A recycling were both regulated by SMPD3, a known regulator of exosome biogenesis.[24] The mechanisms regulating intracellular fetuin-A sorting to MVBs remain to be determined, but we speculate that fetuin-A in MVBs may tether excess calcium to the exosomes destined for secretion,[4,37] and also act to inhibit nucleation of Ca/P in exosomes both intracellularly and in the ECM.[7,34] Although annexin A2 and annexin A6 have previously been implicated in fetuin-A binding and uptake, our data suggest that fetuin-A can be internalized in human VSMCs via a fluid phase uptake mechanism. The scavenger receptor-AI/II has been implicated in the binding and uptake of fetuin-A-containing calciprotein particles by macrophages[38]; however, this receptor was not essential for the uptake of monomeric fetuin-A and calciprotein particles were not present in our experimental conditions.

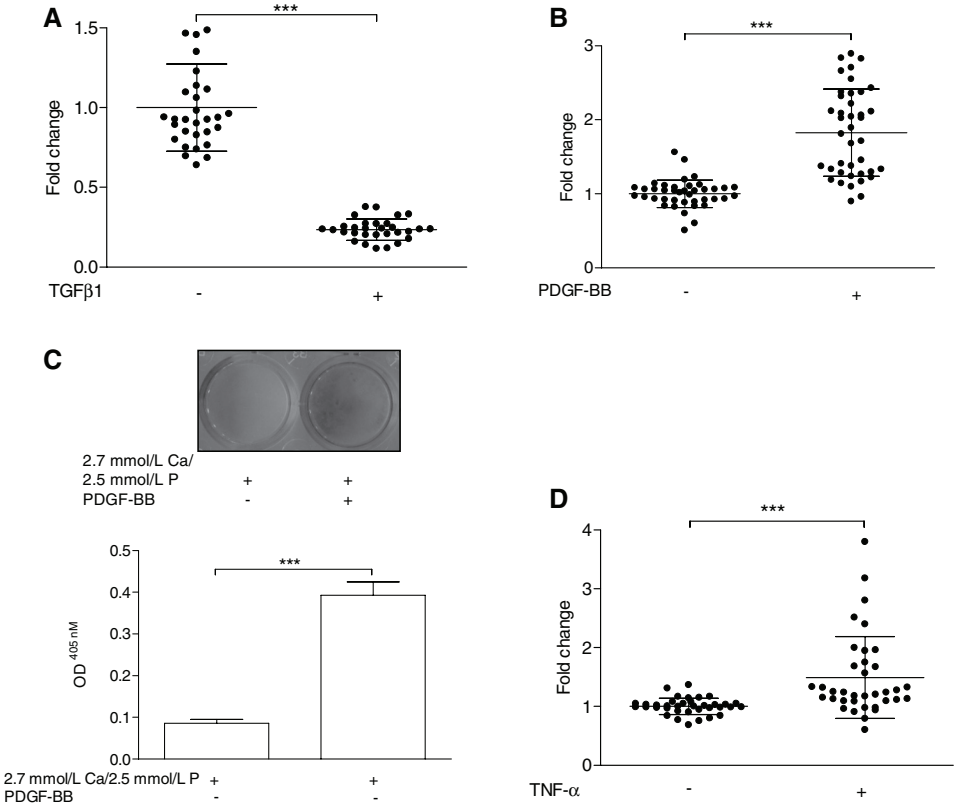

Figure 6. Cytokine and growth factors induce alterations in exosome secretion. **A**, Decreased exosome secretion in response to transforming growth factor (TGF)-β1 treatment (2 ng/mL) for 5 days (mean±SD, N=30). **B**, Vascular smooth muscle cell (VSMC) treatment with platelet derived growth factor-BB (PDGF-BB) (10 ng/mL) in low-serum conditions (0.5% exosome-free fetal bovine serum [FBS]) for 5 days increased exosome secretion quantified using bead capture assay (mean±SD, N=40). **C**, VSMC treatment with PDGF-BB in 0.5% FBS for 48 hours followed by incubation in elevated Ca/P for 24 hours increased calcification as revealed by Alizarin Red staining. **D**, Treatment of VSMCs with tumor necrosis factor (TNF)-α (10 ng/mL) for 5 days resulted in elevated exosome secretion (mean±SD, N=36).

Exosomes Are Novel Players in Calcification

We detected the exosomal marker CD63 in the ECM of precalcified and calcified vessels and showed that exosomes can be extracted from vessels with the earliest detectable calcified particles and before the appearance of overt calcification. In addition, distinct MVB-like organelles were present in VSMCs in vessels from dialysis patients and in response to extracellular calcium. These in vivo findings are consistent with in vitro data showing increased exosome secretion enhanced calcification. Our previous studies showed that VSMC exosomes calcify in vitro in response to calcium, which promotes surface exposure of phosphatidylserine, loss of calcification inhibitors, and loading of exosomes with annexin A6, causing them to become mineralization competent by forming Ca/P nucleation complexes on the inner and outer vesicle membrane.[6] Importantly, calcification in vivo is also associated with increased levels of calcium and a mineral imbalance[4]; in atherosclerotic plaques, extracellular calcium has been measured as high as 30 mmol/L,[39] cell death and release of calcium occur concomitantly with calcification in both the media and intima,[1] while in chronic kidney disease accelerated calcification is associated with elevated levels of calcium and phosphate.[4] Thus, within the pathological vessel wall, exosomes are exposed/released in an environment that can promote their mineralization. Moreover, given that the factors that promote their calcification, such as annexins,[6] are common components of all exosomes, it is likely that exosomes released by any cell type in the vessel wall may have the capacity to calcify.[10] However, the time-course of vesicle mineralization in vivo is unknown.[7,34,35] Although in vitro exosome calcification can be rapidly induced by calcium, it is plausible that exosomes may remain in the vessel wall and calcify over a longer time-course, which is consistent with the heterogeneous deposition of both calcified and noncalcified vesicles at sites of early calcification.[6,34] Proteomics revealed that VSMC exosomes contain a large number of cargo proteins that have been implicated in mineralization, suggesting that exosomes may calcify via additional mechanisms, and this is an area for further investigation.

SMPD3 Is a Key Signaling Molecule in Biomineralization

In VSMCs, we showed that elevated extracellular calcium increased expression of SMPD3 and inhibitors of this pathway blocked exosome secretion. Importantly, we linked exosome secretion to the propensity of VSMCs to calcify. Factors that promoted exosome secretion and SMPD3 activation promoted calcification and inhibitors of SMPD3-blocked calcification.

SMPD3 has previously been implicated in bone mineralization. Fragilitas ossium (*fro/fro*) mice, deficient for the SMPD3 gene, have significant impairment in growth plate structure,[40] and SMPD3 knockdown in osteoblasts prevents mineralization. It was suggested that loss of SMPD3 in bone caused

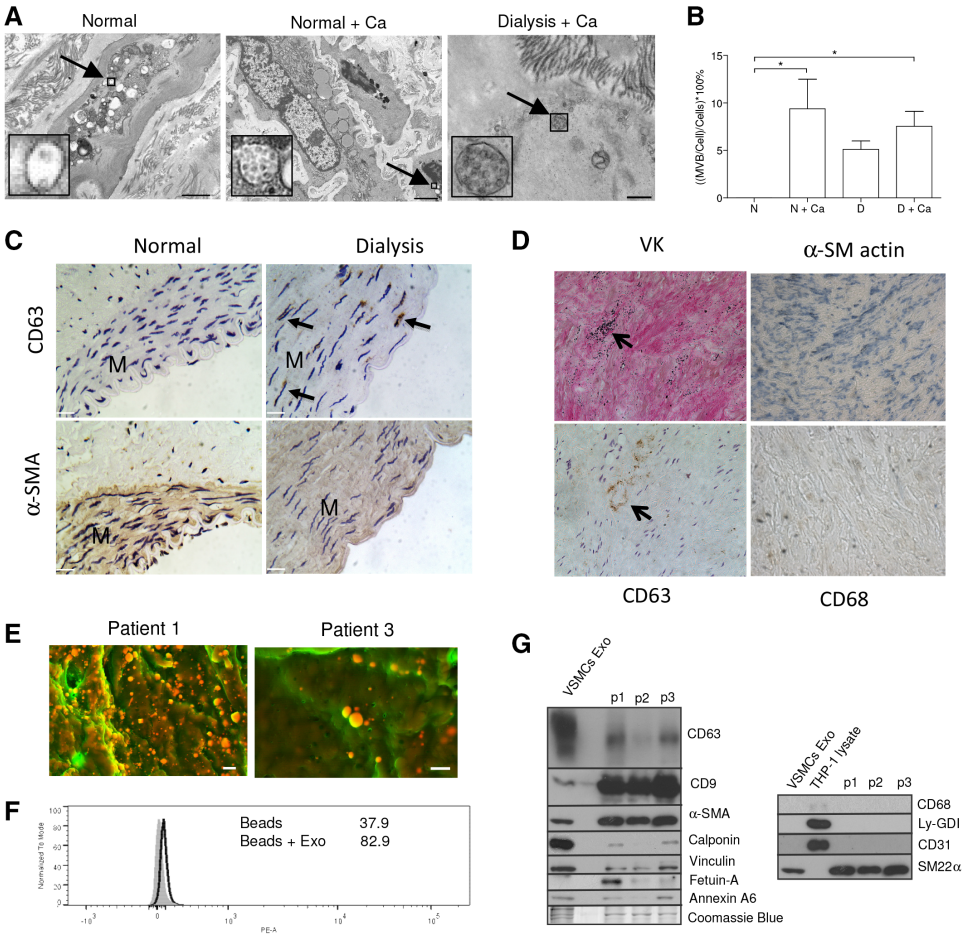

Figure 7. Vascular calcification is associated with the appearance of multivesicular bodies (MVBs) in situ and accumulation of exosomes in vivo. A, Transmission electron microscopy of vessel rings from control and dialysis patients. Panel 1, MVBs were not detected in vascular smooth muscle cells (VSMCs) from normal vessels (Bar, 1 mm). Panels 2 to 3, intracellular MVBs (arrowed) loaded with exosomes were visible in close proximity to the plasma membrane of VSMCs within vessel rings treated in calcifying conditions for 5 days ex vivo. (Bar, 2 and 0.5 μm) Box, Insets showing enlarged MVB compartments, note lack of exosomes in panel 1. **B**, Quantification of MVBs in vessels from normal (N) and dialysis (D) patients treated with or without calcium ex vivo (mean±SEM, N=6–15). **C** and **D**, Deposition of CD63 in arteries. **C**, Calcified artery from 16-year-old child on dialysis showing CD63 staining (arrow) with absence of staining in the normal vessel. **D**, CD63 colocalized with α-smooth muscle actin and calcification in a mildly arteriosclerotic aorta from a 14-year-old child. **E**, Density-dependent color scanning electron micrographs of human aorta showing calcified microparticles. Calcium and phosphate particles are dense as shown by orange color. Scale, 2 μm. **F**, Exosomes from these aortic samples were captured by CD63-beads and detected with PE-CD81 by flow cytometry. **G**, Exosomes from human aorta are enriched with exosomal and VSMC-specific markers but do not contain macrophage, platelet, or endothelial markers.

decreased MV release and defects in ECM composition; however, this was not shown experimentally.[41] These observations suggest that exosome release may also play a role in bone mineralization. Our data showed that osteoblast-derived MVs, particularly those secreted into the media, have many similarities to VSMC exosomes in terms of protein composition and predicted functions, suggesting that at least a subset of bone MVs may also be of exosomal origin.

Limitations and Future Directions

In vitro, we showed that the addition of exosomes to VSMCs, as well as factors that increased exosome production, enhanced calcification. Although some of these factors, such a tumor necrosis factor-α, also act to enhance osteogenic differentiation of VSMCs,[42] other factors, such as platelet derived growth factor-BB do not, suggesting that increased exosome production alone can accelerate mineralization in calcifying conditions. To test this notion more fully, ideally an animal model with decreased exosome production needs to be used. The identification of an important role of SMPD3 in the secretion of exosomes by VSMCs makes *fro/fro* mice a possible model to test the role of exosomes in calcification in vivo and this now needs to be investigated further. However, our findings suggesting that some bone MVs may be exosomal in origin and the observation that *fro/fro* mice have a bone phenotype, suggests that the therapeutic potential of this pathway may be limited, as reducing exosome release/mineralization in VSMCs is likely to also affect bone.

In other cell types, exosomes are known to act in a paracrine and autocrine manner to mediate intercellular transport of bioactive compounds to enhance cellular processes such as migration.[43] Our proteomics data suggested that VSMCs may also have such roles beyond calcification, and we showed that exosome production was increased in proliferative VSMCs. If exosome release at sites of vascular injury primes the vessel wall for calcification when conditions favor mineralization, it may be

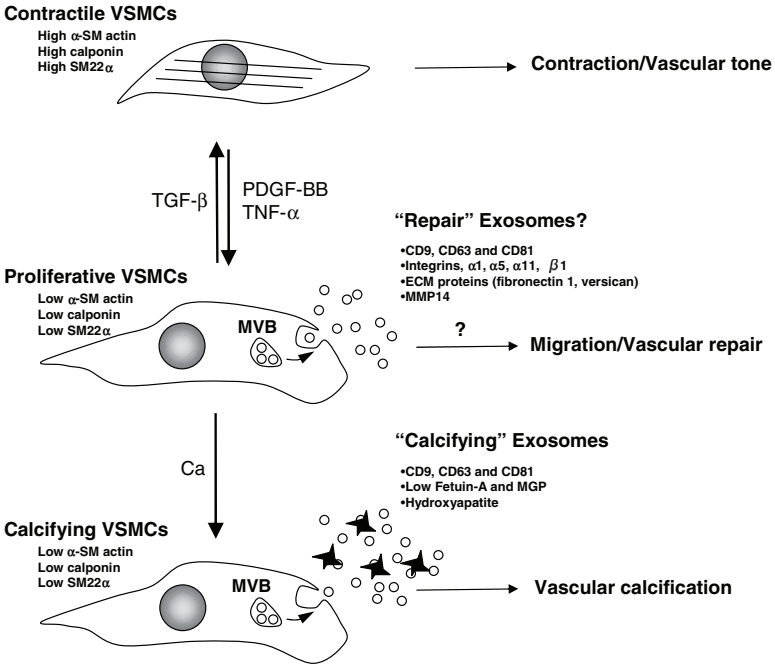

Figure 8. Proposed role of exosomes in vascular repair and calcification. Healthy contractile vascular smooth muscle cells (VSMCs) maintain vascular tone but injury causes a phenotypic transition and proliferation. Proliferative VSMCs actively release CD63/CD81-positive exosomes enriched with calcification inhibitors such as fetuin-A and other cargoes that may facilitate vascular repair processes such as adhesion and migration. Prolonged stress and a mineral imbalance enhance exosome release and shift them toward a procalcific state.

possible to target directly factors that promote exosome release. However, only some inflammatory mediators enhanced exosome production, others decreased their production, suggesting that further work is required to identify context-specific pathways regulating exosome release and ultimately mineralization.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Vascular calcification is a risk factor for high cardiovascular morbidity and mortality.
- Vascular smooth muscle cells mediate vascular calcification.
- Calcification is initiated in matrix vesicles (MV), membrane-enclosed vesicles or unknown origin, secreted by vascular smooth muscle cells and loaded with calcification inhibitors.

What New Information Does This Article Contribute?

- The circulating calcification inhibitor fetuin-A is released in exosomes identifying MVs as exosomal in origin.
- Vascular smooth muscle cell–derived exosomes contain proteins involved in calcification as well as cell migration and adhesion.
- Exosome secretion is regulated by sphingomyelin phosphodiesterase 3.
- Inhibition of exosome release blocks calcification.

Vascular smooth muscle cells secrete MVs, which form the nidus for hydroxyapatite deposition in the vessel wall. However, the factors that regulate MV biogenesis, cargo loading, and release are poorly understood. We used the circulating calcification inhibitor fetuin-A as a tracer to identify the origin of MVs and found that it is trafficked and released via the exosome pathway. Exosome secretion was regulated by sphingomyelin phosphodiesterase 3 and was increased in response to stresses promoting calcification. We detected amorphous hydroxyapatite in calcifying exosomes and specific inhibition of exosome release blocked calcification. Exosomes were detected in the vasculature in vivo and their presence was associated with calcification. Proteomics revealed that in addition to their role in pathological calcification, vascular smooth muscle cell–derived exosomes may also function during vascular repair and mediate processes such as adhesion and migration. Modulation of regulatory pathways involved in exosome secretion, as well as loading with biologically active cargoes such as calcification inhibitors, may provide novel therapeutic targets to counteract the onset of vascular calcification.