Circulation Research
Journal of the American Heart Association

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Circ. Res. 2011;109;e1-e12; originally published online May 12, 2011;
DOI: 10.1161/CIRCRESAHA.110.238808
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Calcium Regulates Key Components of Vascular Smooth Muscle Cell–Derived Matrix Vesicles to Enhance Mineralization

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Rationale: **Matrix vesicles (MVs) are specialized structures that initiate mineral nucleation during physiological skeletogenesis. Similar vesicular structures are deposited at sites of pathological vascular calcification, and studies in vitro have shown that elevated levels of extracellular calcium (Ca) can induce mineralization of vascular smooth muscle cell (VSMC)–derived MVs.**

Objectives: **To determine the mechanisms that promote mineralization of VSMC-MVs in response to calcium stress.**

Methods and Results: **Transmission electron microscopy showed that both nonmineralized and mineralized MVs were abundantly deposited in the extracellular matrix at sites of calcification. Using cultured human VSMCs, we showed that MV mineralization is calcium dependent and can be inhibited by BAPTA-AM. MVs released by VSMCs in response to extracellular calcium lacked the key mineralization inhibitor matrix Gla protein and showed enhanced matrix metalloproteinase-2 activity. Proteomics revealed that VSMC-MVs share similarities with chondrocyte-derived MVs, including enrichment of the calcium-binding proteins annexins (Anx) A2, A5, and A6. Biotin cross-linking and flow cytometry demonstrated that in response to calcium, AnxA6 shuttled to the plasma membrane and was selectively enriched in MVs. AnxA6 was also abundant at sites of vascular calcification in vivo, and small interfering RNA depletion of AnxA6 reduced VSMC mineralization. Flow cytometry showed that in addition to AnxA6, calcium induced phosphatidylserine exposure on the MV surface, thus providing hydroxyapatite nucleation sites.**

Conclusions: **In contrast to the coordinated signaling response observed in chondrocyte MVs, mineralization of VSMC-MVs is a pathological response to disturbed intracellular calcium homeostasis that leads to inhibitor depletion and the formation of AnxA6/phosphatidylserine nucleation complexes. (*Circ Res*. 2011;109:e1-e12.)**

Key Words: matrix vesicles ■ annexin ■ calcification ■ vascular smooth muscle cells ■ calcium ■ proteomics

Vascular calcification is the deposition of apatite mineral in the medial or intimal layers of the vessel wall and is a clinically significant pathology in atherosclerosis, diabetes, chronic kidney disease, and aging. Once established, vascular calcification is progressive, particularly in association with raised levels of extracellular mineral ions such as calcium and phosphate.[1] Recent nuclear magnetic resonance studies have shown that the structural organization of the molecular components of vascular mineralizations are identical to those in bone.[2,3] This implies mechanistic similarities during the earliest phases of initiation of mineral nucleation in both tissues.

During developmental osteogenesis/chondrogenesis, specialized membrane-bound bodies called matrix vesicles (MVs), which originate from the plasma membrane of chondrocytes and osteoblasts, serve as nucleation sites for hydroxyapatite.[4] In cartilage, MV production occurs throughout the growth plate, but MVs are "mineralization competent" only in the hypertrophic zone.[4] This transition is induced by an intracellular calcium signal that initiates changes in gene transcription and the subsequent release of MVs that are able to nucleate mineral to form hydroxyapatite nanocrystals.[5] Mineralization-competent MVs are enriched with the calcium-binding annexins (Anx) A2, A5, and A6 and surface

Original received December 8, 2010; revision received April 28, 2011; accepted May 2, 2011. In March 2011, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.2 days.

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Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.110.238808

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Non-standard Abbreviations and Acronyms	
ALP	alkaline phosphatase
AnxA2	annexin A2
AnxA5	annexin A5
AnxA6	annexin A6
BAPTA-AM	1,2-bis-(o-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester
ECM	extracellular matrix
MV	matrix vesicle
MGP	matrix Gla protein
VDAC1	voltage-dependent anion-selective channel protein 1

alkaline phosphatase (ALP), which releases phosphate by degrading pyrophosphate, a potent inhibitor of hydroxyapatite crystal growth; however, the exact mechanisms whereby MVs concentrate calcium and phosphate to enable mineral nucleation have not been resolved completely. A number of additional factors have been implicated, including sodium-dependent phosphate transporters and phospholipid components of the vesicle membrane such as phosphatidylserine.[5–8]

Ultrastructural studies have identified hydroxyapatite-containing membrane vesicles in the vessel wall, which suggests that these structures may also provide the first nidus for vascular calcification.[9,10] Vascular smooth muscle cells (VSMCs) play a key role in initiating and regulating vascular calcification, and at sites of calcification, they undergo an osteocytic/chondrocytic phenotypic change and upregulate expression of mineralization-regulating proteins that are normally confined to bone and cartilage.[11] Concomitant with this phenotypic transition, in vitro VSMCs also spontaneously release membrane-bound vesicles.[12] Under normal conditions, these VSMC-derived vesicles do not calcify, because they are loaded with mineralization inhibitors such as matrix Gla protein (MGP) and fetuin-A, which act to block mineral nucleation.[12] However, our previous studies have shown that treatment of VSMCs with elevated levels of extracellular calcium can stimulate the production of calcifying vesicles that contain preformed apatite, a hallmark of mineralization-competent MVs.[12,13] Importantly, elevated extracellular phosphate could not induce the same effect on MV mineralization, which suggests that calcium uptake, raised intracellular calcium, or both may trigger VSMC MV release and mineralization. Indeed, elevated extracellular or intracellular calcium has been widely reported in pathologies associated with increased vascular calcification.[10,14,15] Thus, the local environment of MV biogenesis is likely to affect their ability to calcify, and this notion is supported by studies showing that vesicles isolated from the normal vessel wall are inefficient at accumulating calcium compared with those from calcified or atherosclerotic regions.[9]

To identify the mechanisms of elevated calcium-induced MV mineralization, we used proteomics and molecular approaches to characterize the properties of VSMC-derived MVs (VSMC-MVs) released under basal noncalcifying conditions and in response to raised extracellular mineral ions. We show that key events in VSMC-MV calcification are calcium-induced loss of inhibitors and membrane association of AnxA6 and phosphatidylserine to form a complex that can nucleate hydroxyapatite. Alteration of the properties and biogenesis of VSMC-derived vesicles may be a useful strategy for limiting vascular calcification.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Extracellular Mineral Ion-Induced VSMC Calcification

Explanted cultures of human aortic VSMCs were established as described previously[12] and used between passages 4 and 12. VSMCs were treated with calcification media supplemented with calcium (5.4 mmol/L) or calcium/phosphate (2.7 mmol/L calcium, 2.5 mmol/L phosphate) as indicated and ^{45}Ca (≈50 000 cpm/mL) as described previously[12] and exposed for between 16 hours and 5 days in the presence of 0.5% BSA. One to 5 μmol/L BAPTA-AM (1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) was added at the time of calcium addition. Calcification was quantified by liquid scintillation counting for ^{45}Ca and visualized by Alizarin Red staining.

Preparation of Vesicles and Assays

Apoptotic bodies and MVs were isolated by differential centrifugation as described previously, and MV calcification potential was measured by ^{45}Ca incorporation as described previously.[12,16] ALP activity was measured with a *p*-nitrophenyl phosphate substrate (Sigma, St Louis, MO). Protein content was measured with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA). Matrix metalloproteinases (MMPs) were measured with zymography or fluorescence assay kits according to the manufacturer's instructions.

Protein Analysis of VSMC-MVs by Mass Spectrometry

MVs were resuspended in sample buffer, proteins were separated on a 4% to 15% gradient SDS-PAGE gel (Bio-Rad) and stained with Colloidal Blue (Invitrogen, Carlsbad, CA), and protein bands were excised. Excised proteins were identified with liquid chromatography tandem mass spectrometry, and the data were analyzed with the Mascot program (Matrix Science Inc, Boston, MA).

Two-dimensional difference gel electrophoresis was performed as described previously[17] with modifications. Briefly, MV proteins (100 μg) were labeled with Cy3 DIGE Fluor minimal dye (GE Healthcare, Little Chalfont, United Kingdom) and separated by 2-dimensional difference gel electrophoresis, and proteins were silver stained with a Plus One silver stain kit with modification (Amersham, Uppsala, Sweden). Proteins were excised and identified by liquid chromatography tandem mass spectrometry. Detailed protocols for 2-dimensional gel electrophoresis and silver staining are provided in the online-only Data Supplement and online at http://www.vascular-proteomics.com.

Antibodies

Antibodies used were AnxA2 (BD Transduction Laboratories, San Diego, CA), AnxA5 (Abcam, Cambridge, United Kingdom), AnxA6 (BD Bioscience, San Diego, CA), vinculin (Sigma), MGP (a monoclonal antibody raised against aa 3-15), phosphatidylserine antibody (Abcam, Cambridge, United Kingdom), antibody for the extracellular domain of integrin β1 (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa Fluor 488 goat anti-mouse antibody for immunofluorescence and flow cytometry (Invitrogen, Eugene, OR), and horseradish peroxidase–conjugated secondary antibody for immunohistochemistry (GE Healthcare, Little Chalfont, United Kingdom).

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Biotin Cross-Linking

VSMC surface labeling was performed with a Pierce cell-surface protein isolation kit (Pierce, Rockford, IL) according to the manufacturer's protocol.

Small Interfering RNA Transfection

Small interfering RNA oligonucleotides targeting annexins predesigned by Qiagen (Valencia, CA) were transfected into VSMCs with HiPerFect (Qiagen) according to the manufacturer's protocol. VSMCs were transfected for 48 hours before addition of calcification medium that contained 1 μmol/L fetuin for a further 72 hours before analysis.

Flow Cytometry

A total of 1×10^5 VSMCs were incubated with primary antibody or the corresponding isotype-matched IgG control for 30 minutes at 4°C. After they were washed, VSMCs were incubated with secondary antibody for 30 minutes at 4°C, washed again, and analyzed with a BD FACScalibur (BD Bioscience). MVs were coupled to 4-μm surfactant-free aldehyde/sulfate latex beads (Invitrogen) as described previously.[18] As a control, 20 μL of beads was incubated with antibody and analyzed as above.

Biochemical Analysis of MVs

An aliquot of VSMC-MVs (5 μg) was treated with EGTA 10 mmol/L for 15 minutes at room temperature. MV membranes and extracted proteins were separated by ultracentrifugation at 100 000*g* for 30 minutes, and the pelleted membrane fraction was resuspended. Samples were then treated with or without EGTA 10 mmol/L or $CaCl_2$ 2 mmol/L and disrupted by freeze/thaw 4 times. Membrane and luminal extracted proteins were isolated by centrifugation as before, and the resultant pellet was resuspended. The fractions were analyzed by 10% (wt/vol) SDS-PAGE and Western blot.

Statistical Analysis

Data were analyzed by 1-way ANOVA with Bonferroni post hoc test or *t* test as indicated with PRISM software (GraphPad, San Diego, CA). Data show the mean±SD. $P<0.05$ was considered statistically significant.

Results

Vesicles Mediate Calcification In Vivo

Transmission electron microscopy was used to examine MV deposition in normal and calcified arteries in different disease contexts. In young healthy arteries, numerous MVs 50 to 500 nm in size were observed in the extracellular matrix (ECM) of the vessel media; however, there was no evidence of crystalline mineral within these structures (Figure 1A). In minimally calcified arteries from patients with atherosclerosis and chronic kidney disease, mineralization was observed in a subset of MVs that clustered in association with elastin and collagen fibrils; however, mineralization did not extend into these ECM components (Figures 1B–D). Only in more heavily calcified vessels obtained from diabetic patients was mineralization observed to extend into the ECM and involve collagen; however, in these vessels, mineralization was also apparent on membrane surfaces, including the inner and outer membranes of MVs in close proximity to collagen and on the VSMC plasma membrane (Figure 1E). These observations suggest that mineral is nucleated on membranes and progresses to the ECM after initiation in MVs, a subset of which become mineralization competent in diseased arteries.

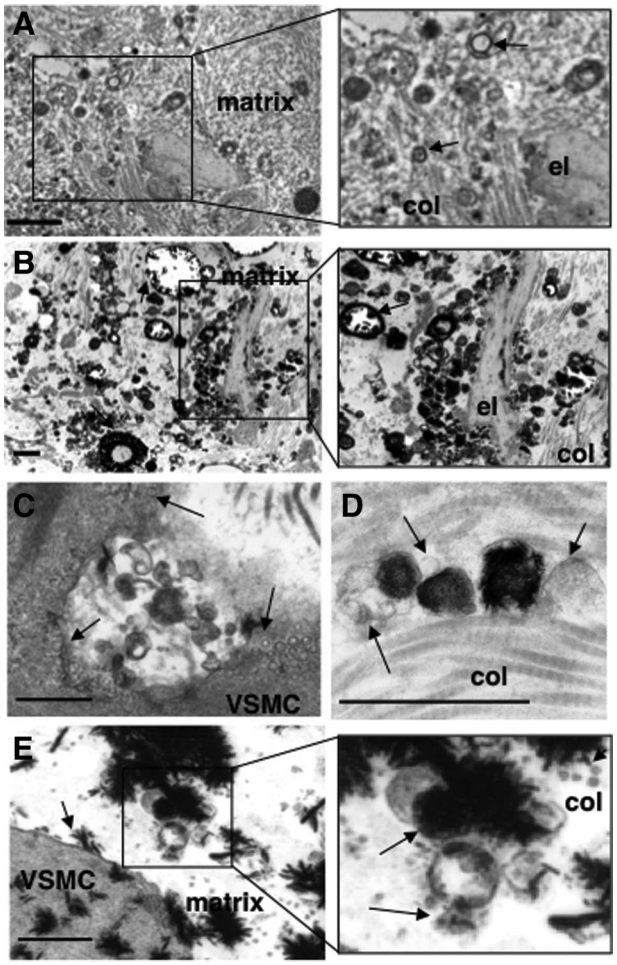

Figure 1. MV deposition in the ECM in vivo. Transmission electron microscopy shows accumulation of numerous vesicles in the ECM of normal (**A**) and calcified (**B–E**) vessels. **A**, Distal abdominal aorta from a 24-year-old woman with no gross or histological calcification. Noncalcified MVs (**arrows**) lacking hydroxyapatite crystals and varying in size and appearance were associated with elastin and collagen in the normal media. **B**, Distal abdominal aorta from a 60-year-old woman with mild atherosclerosis and focal calcification. Calcified MVs associated with elastin and collagen in the media of a calcified atherosclerotic artery. **C** and **D**, Renal artery from an 8-year-old girl on dialysis. Noncalcified MVs were in close proximity to the plasma membrane (arrowed) of a VSMC. **D**, Higher-power view from the same patient highlighting the presence of both calcified and noncalcified vesicles (**arrows**) abutting collagen fibrils. Note that the mineral did not involve the ECM. **E**, A heavily calcified femoral artery from a 58-year-old diabetic patient showing mineralization on the VSMC plasma membrane (**arrow**) and on collagen (**arrowhead**). Note the presence of MVs in the ECM and in association with collagen. Some mineral on these MVs was on the external membrane and extended into the ECM (**arrows**). el indicates elastin; col, collagen. **Scale bar**, 1.0 μm.

Elevated Extracellular Calcium Induces Alterations in Cytosolic Ca^{2+} Levels to Promote MV-Mediated Mineralization of VSMCs

Our previous studies showed that mineralization-competent MVs were released by VSMCs only in response to elevated extracellular calcium,[5,12] and we found that blocking calcium uptake by VSMCs also inhibited VSMC calcification (Online Figure I). Calcium entry should lead to alterations in cytosolic calcium, and treatment of VSMCs with the intracellular

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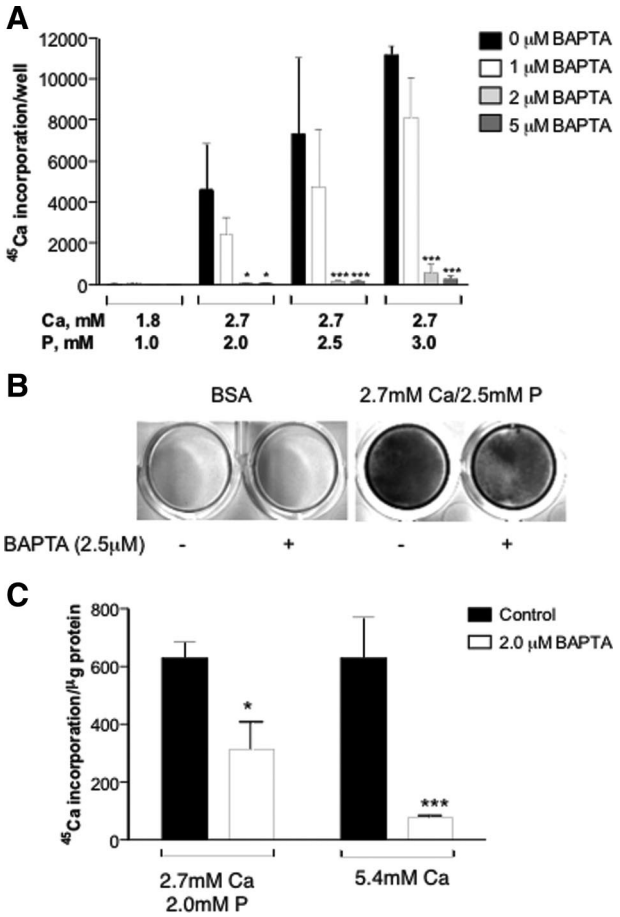

Figure 2. Reducing intracellular calcium ion concentration inhibits MV mineralization. A, Graph showing the dose-dependent inhibitory effect of BAPTA-AM on VSMC calcification. VSMCs were treated in serum-free media for 16 hours followed by 24 hours of treatment with or without BAPTA-AM in calcifying conditions as indicated. Mean±SD, n=3. *$P<0.05$, ***$P<0.001$ (ANOVA). **B**, Alizarin Red staining showing reduced calcification in BAPTA-AM–treated VSMCs. **C**, MVs isolated from VSMCs treated with calcifying media for 16 hours in the presence of 2 μmol/L BAPTA-AM showed reduced calcification potential, shown by reduced ^{45}Ca uptake. Mean±SD (n=3). *$P<0.05$ and ***$P<0.001$ compared with control.

calcium chelator BAPTA-AM in the presence of calcifying media decreased VSMC calcification in a dose-dependent manner (Figures 2A and 2B). Moreover, using a quantitative in vitro calcification assay, we found that MVs isolated from BAPTA-AM–treated VSMCs had a significant reduction in their capacity to mineralize (Figure 2C), which suggests extracellular calcium uptake and cytosolic calcium alterations are necessary for the production of mineralization-competent MVs.

Calcium Does Not Increase ALP in VSMC-MVs but Induces Loss of the Inhibitor MGP and Activation of MMP2

Next, we assessed the similarities between VSMCs and chondrocyte-derived MVs with respect to key regulators of mineralization. ALP is enriched in chondrocyte MVs[4]; however, in comparison, VSMCs show extremely low levels of activity of this enzyme. Although there was some enrichment of ALP in VMSC-MVs, ALP activity was decreased after treatment with extracellular calcium (Figure 3B). Treatment with BAPTA-AM failed to induce changes in ALP activity (data not shown), which suggests it is not a key calcium-responsive mediator of MV calcification in VSMCs.

MGP is a key inhibitor of MV calcification in both chondrocytes and VSMCs.[19] Western blots showed that MVs isolated from VSMCs treated with calcifying media had a dramatic reduction in MGP content 48 hours after treatment (Figure 3B). To test the factors that contributed to this loss, we treated VSMCs with elevated phosphate or calcium alone. Treatment of VSMCs with phosphate had no effect on MGP content of MVs (data not shown). Treatment with elevated calcium induced an initial increase in MGP loading at 24 hours, but after 48 hours, MGP was significantly reduced in mineralization-competent MVs. This pattern of MGP loss was also mirrored in whole-cell lysates (Online Figure II).

MVs associated with ECM components, and previous studies have shown that elastin degradation promotes calcification.[20] Zymography and fluorimetric assays showed that VSMC-MVs were enriched in MMP2 but contained no MMP9, whereas the majority of cathepsin S activity was present in apoptotic bodies (Online Figure III). Calcium treatment maximally activated MMP2, as indicated by no increased activation after treatment with APMA (4-aminophenylmercuric acetate), and calcium-dependent MMP2 activation could be blocked by treatment of VSMCs with BAPTA-AM (Figures 3C and 3D; Online Figure III). Treatment of VSMCs with the MMP inhibitor GM6001 dose dependently decreased calcification in response to calcium/phosphate after 16 hours (Figure 3E).

Proteomic Characterization of Mineralization-Competent MVs Reveals a Role for Annexins

To identify additional mediators of calcification, we analyzed the composition of VSMC-MVs using protein mass spectrometry (Online Figures IV and V). Two approaches were used: (1) 1-dimensional gel electrophoresis to isolate MV components concentrated in response to calcium, and (2) CyDye tagging and 2-dimensional gel comparison to identify membrane components of MVs. The 79 identified proteins were broadly classified into 10 major groups (Online Tables I and II): 12 proteins potentially involved in mineralization processes, including calcium channels and ECM components; 24 proteins possibly related to MV biogenesis, including cytoskeletal proteins and proteins involved in intracellular vesicle trafficking; 15 different stress-related proteins, mainly involved in oxidative stress and protein folding; and 5 serum proteins. Comparative proteomic bioinformatics analysis identified 38 proteins that had been identified previously in chondrocyte MVs[7,8] (Table). Of particular note was the identification of annexins, which have been shown to specifically accumulate in chondrocyte-derived mineralization-competent MVs in a calcium-dependent manner.[5]

AnxA6 Is a Biomarker of Vascular Calcification and Is Required for MV Mineralization

Western blots showed that only AnxA2, A5, and A6 were selectively enriched in MVs compared with VSMC lysates and apoptotic bodies, which implies they may play a specific

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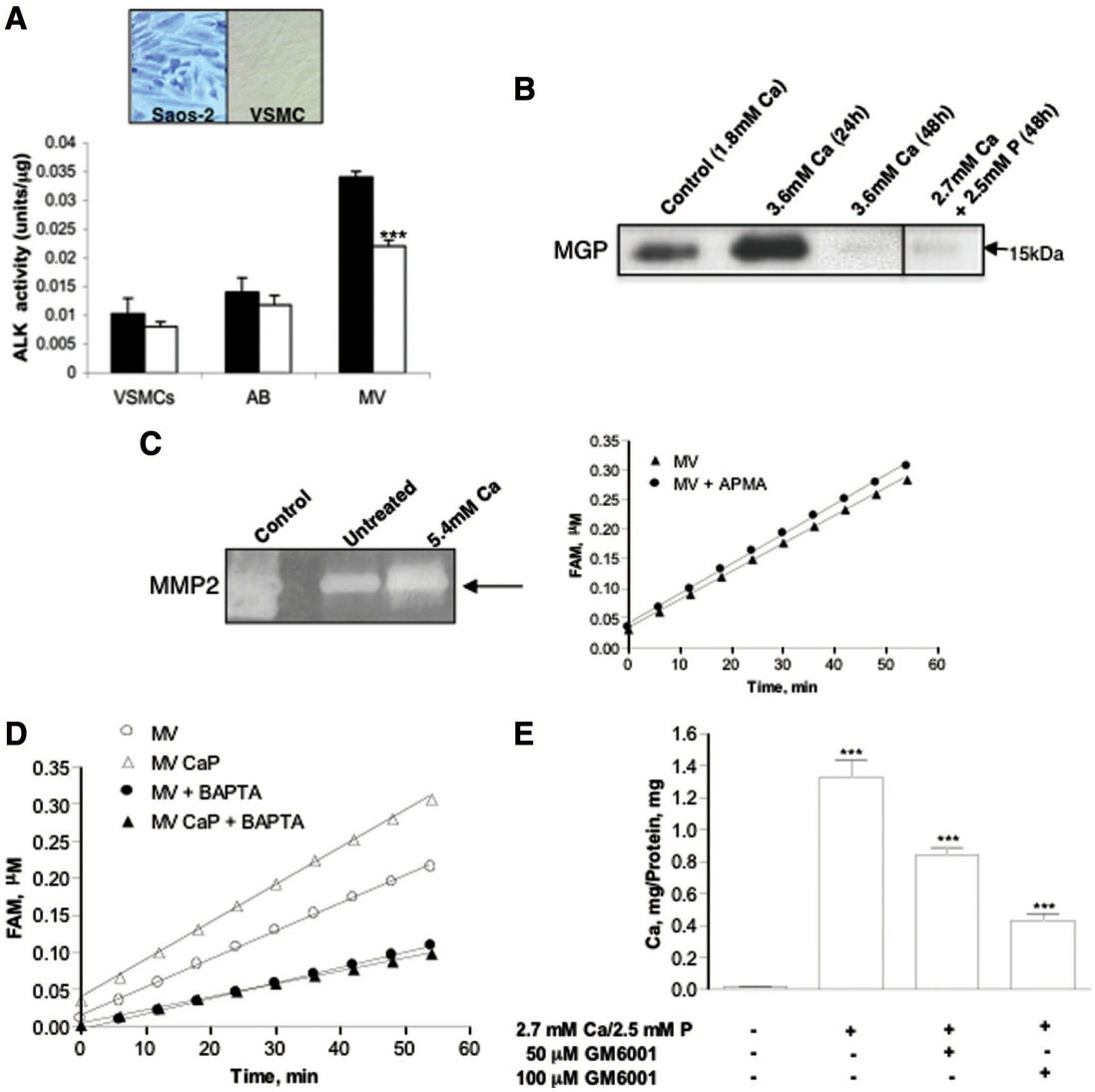

Figure 3. Calcium reduces ALP activity, triggers loss of MGP, and activates MMP2 in MVs. A, VSMCs exhibited very low ALP (=ALK) activity compared with bone cells Saos-2 (1.0±0.001 units/μg vs 91.4±21.6 units/μg). ALP activity is also shown with blue stain. ALP activity was enriched in MVs compared with VSMCs or apoptotic bodies (AB) but was decreased significantly by treatment in calcifying media (2.7 mmol/L calcium/2.0 mmol/L phosphate). n=4. ***$P<0.0001$. **B**, Western blot showing that MGP was initially increased in MVs in response to calcium but was subsequently reduced to barely detectable levels after longer-term treatment. **C**, Zymography showing MV enrichment with MMP2. Treatment of VSMCs with calcium increased MMP activation in MVs (**arrow**), and fluorimetric kinetic analysis of MMP2 activity in MVs from calcium-treated VSMCs in the presence or absence of the activator APMA indicated that activation was maximal (FAM=5-FAM, 5-carboxyfluorescein). **D**, Kinetic analysis showed that MMP2 activity was higher in calcifying MVs than in control MVs. Treatment of VSMCs with BAPTA-AM attenuated MV-associated MMP2 activity. **E**, Treatment of VSMCs with the MMP inhibitor GM6001 in the presence of calcifying media dose dependently inhibited calcification after 16 hours. n=5. ***$P<0.0001$ (ANOVA).

role in MVs and not in other membrane-derived vesicles (Figure 4A). Treatment of VSMCs with calcifying media induced selective enrichment of AnxA6, whereas levels of AnxA2 and A5 remained relatively unchanged or were even reduced (Figure 4B).

The enrichment of AnxA6 in calcifying MVs was supported by immunohistochemistry of calcified human arteries (Figure 4C), which showed a significant deposition of AnxA6 at sites of calcification. In normal noncalcified arteries, AnxA5 and A6 were undetectable, whereas AnxA2 was detectable in VSMCs. In calcified arteries, AnxA2 staining remained unchanged, whereas some patchy AnxA5 staining was evident in association with VSMCs. AnxA6 staining was markedly upregulated, colocalized consistently with von Kossa–positive areas, and was deposited within the ECM surrounding calcified VSMCs. To evaluate the significance of annexins in VSMC mineralization, we depleted AnxA2, A5, and A6 using specific small interfering RNAs and found that only AnxA6 knockdown reduced calcification, which suggests a crucial role for AnxA6 in vascular mineralization (Figures 4D–F).

AnxA6 Translocates to the Plasma Membrane in Response to Cytosolic Calcium Elevation

Annexins are normally cytosolic, but in response to a rise in extracellular calcium, they can translocate to the plasma membrane from which MVs are shed.[4,5] Immunofluorescence staining of VSMCs confirmed that AnxA6 was cytosolic under normal conditions (Figure 5A). In response to calcium,

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Table. Common Components Identified in Both VSMCs and Bone-Derived MVs

Putative Ca channels	Cytoskeleton proteins
Annexin A1	Myosin light polypeptide 6
Annexin A2	Moesin
Annexin A4	Actin, β
Annexin A5	14-3-3 protein ε
Annexin A6	F-actin–capping protein subunit α1
VDAC1	Tropomyosin, α3 chain
Putative phosphate source	Tropomyosin, α4 chain
5′ nucleotidase, ecto	Vimentin
ECM mineralization	Plasma membrane proteins
Collagen, type VI, α1 precursor	Aminopeptidase N
Collagen, type I	Integrin, α3
Galectin-1	Integrin, αV
Oxidative stress and protein folding	Integrin, β1
Peroxiredoxin 1	Na^+/K^+ ATPase, α1
Peroxiredoxin-2	Intracellular proteins
Glutathione S-transferase P1	α-Enolase
Peptidyl-prolyl cis-trans isomerase A	L-lactate dehydrogenase A chain
Calreticulin	Nucleoside diphosphate kinase A
Serum proteins	Elongation factor 1, α1
α-2-HS-glycoprotein	Elongation factor 1, β
Apolipoprotein A-I	Eukaryotic translation initiation factor 5A-1
α2 Macroglobulin	
Fibronectin	

Proteins were identified using 1- or 2-dimensional electrophoresis and liquid chromatography tandem mass spectrometry of VSMC-derived MVs. The VSMC protein list was compared with proteomic analysis of previously published bone-derived matrix vesicle data.[7,8]

AnxA6 fluorescence became stronger at the plasma membrane and was enriched on the membrane of small vesicles, apparently budding from the plasma membrane surface. In contrast, localization and levels of AnxA2 and A5 were unchanged in response to calcium (Online Figure VI). Translocation of AnxA6 to the plasma membrane in response to calcium and inhibition of this translocation with BAPTA-AM were confirmed by biotin cross-linking and flow cytometry (Figures 5B–D), which supports the notion that AnxA6 enrichment at the plasma membrane is calcium dependent.

Annexin A6 Localizes to the Membrane of MVs in Response to Calcium

We next examined the localization of AnxA6 within MVs using flow cytometry. Using the cell-surface marker integrin-β1, we established that the orientation of the MV membrane was similar to the plasma membrane (Figure 6A). Annexins were also detected on the outer surface of the MV membrane; however, only AnxA6 was increased on the surface in response to calcium treatment (Figure 6A). To examine Anx6 localization further, EGTA was used to extract calcium-dependent binding of AnxA6 from the outer surface of MVs. This treatment released AnxA6 from the surface of calcifying MVs, whereas a significantly lower amount of AnxA6 was on the outer surface of control vesicles (Figure 6B). Next, to elute AnxA6 from the inner surface of MVs, we disrupted the membrane using a freeze/thaw cycle and again extracted with EGTA. We observed a significant extraction of AnxA6 from the inner membrane of MVs. In contrast, AnxA2 was only extracted by EGTA from the inner membrane (Figure 6B). As a further control, the intraluminal localization of AnxA2 in MV was confirmed with 2-dimensional difference gel electrophoresis proteomics (Figure 6C; Online Figure II).

We also found that a high proportion of AnxA2 and A6 was not extractable by EGTA, which suggests a tight calcium-independent incorporation within the membrane bilayer (Figure 6B). This localization has been associated with AnxA5 calcium-channel activity in chondrocyte MVs.[21] However, treatment of VSMCs and VSMC-MVs with the annexin calcium-channel blocker K201 did not reduce calcification of VSMCs or isolated MV nor did treatment with antibodies specific for AnxA2 or A6,[21] which suggests that annexins do not act as calcium channels in VSMC-MVs (Online Figure VII).

Calcium-Dependent Phosphatidylserine-Annexin Complexes Mediate MV Calcification

It is known that annexin binding to the plasma membrane is mediated by negatively charged phospholipids, in particular phosphatidylserine.[22] Moreover, complexes of annexins with phosphatidylserine have been shown to possess strong nucleation activity and significantly enhance crystalline mineral formation in chondrocyte MVs.[6,21] Therefore, an accumulation of AnxA6 on the outer surface of MVs may occur simultaneous with externalization of phosphatidylserine in response to high calcium, leading to the formation of nucleation sites. Indeed, treatment of VSMCs in calcifying conditions resulted in the upregulation of phosphatidylserine on the VSMC surface (Figure 7A). This redistribution was calcium dependent and inhibited by BAPTA-AM (Figure 7B). Flow cytometry confirmed that phosphatidylserine was also present on the MV surface and was upregulated in the presence of calcifying media, and this phosphatidylserine externalization was calcium dependent and again inhibited by BAPTA-AM (Figure 7B; Online Figure VIII).

Mineralization-competent MVs were visualized with transmission electron microscopy to examine the sites of crystalline hydroxyapatite accumulation. MVs ranging in size from 50 to 500 nm showed accumulation of crystalline mineral on the surface and within the lumen (Figure 7C), consistent with nucleation complexes of AnxA6 and phosphatidylserine being present on both the inner and outer membranes of MVs (Figure 7D).

Discussion

Mineralization-Competent VSMC-MVs Share Similarities With Chondrocyte-Derived MVs

In the present study, we have shown that VSMC calcification is first initiated in extracellular MVs. In vitro studies have demonstrated that MV calcification is induced by calcium-dependent loss of MGP loading and the concomitant upregulation and redistribution of phosphatidylserine and Anx6 complexes that act to nucleate hydroxyapatite on the inner

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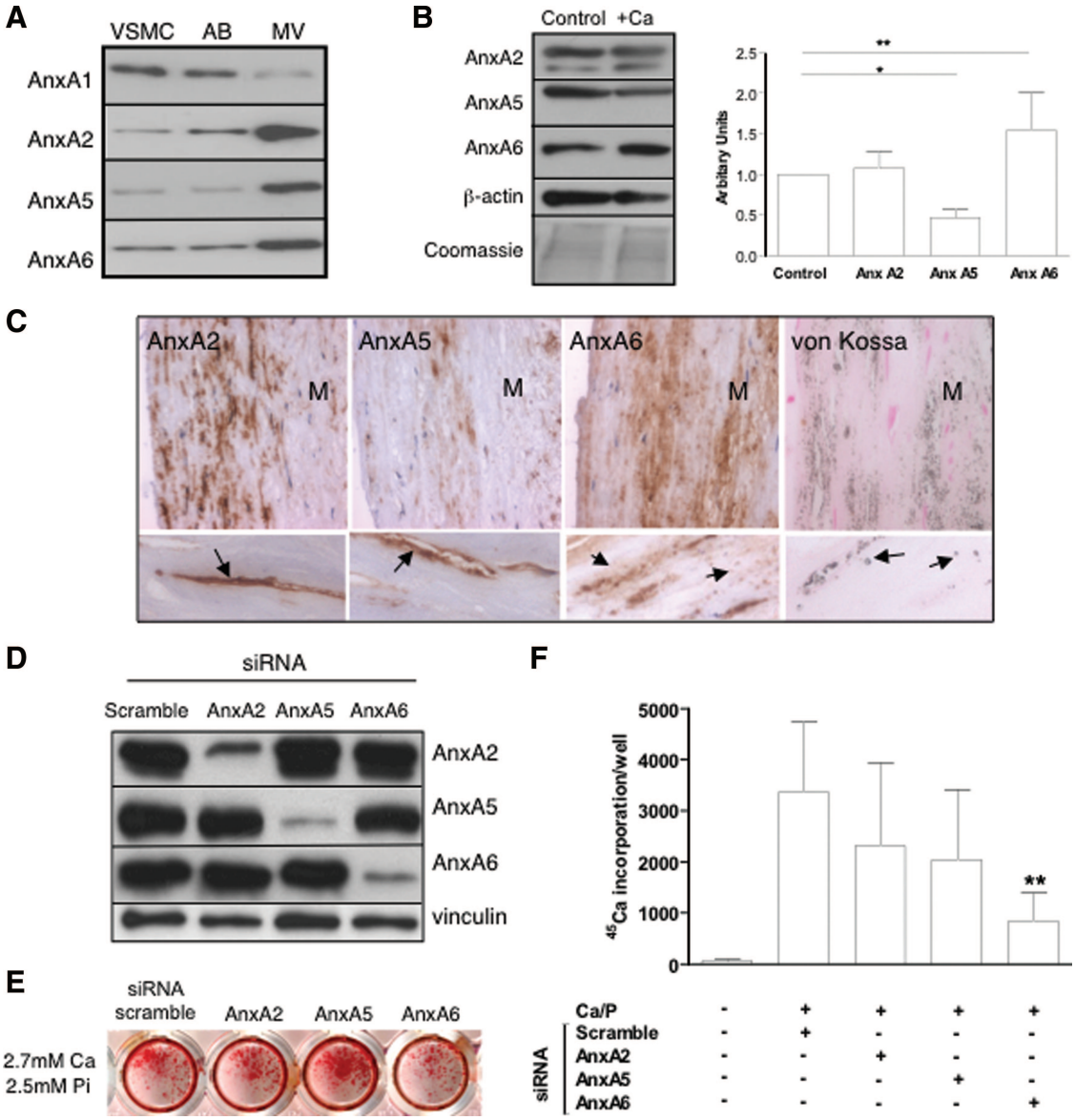

Figure 4. VSMC calcification is mediated by AnxA6. A, Western blot showing enrichment of AnxA2, A5, and A6 in MVs compared with apoptotic bodies (AB) and VSMCs. **B**, Western blot showing enrichment of AnxA6 in mineralization-competent MVs (+Ca, 2.7 mmol/L calcium/2.5 mmol/L phosphate), whereas AnxA2 and A5 were unchanged or reduced. Graph shows quantitation of n=6 experiments. ANOVA: $^{*}P<0.05$, $^{**}P<0.001$. **C**, Immunohistochemistry of adjacent sections of a calcified matrix-rich region of an atherosclerotic plaque showing colocalization of AnxA6 with calcification (von Kossa stain). AnxA2 was associated with VSMC membrane (**arrow**) and was not observed in the matrix. AnxA5 was also associated with the VSMC membrane (**arrow**) and was not abundant in calcified areas. In contrast, AnxA6 was deposited in association with calcified VSMCs and within the ECM (**arrows**) in vesicle-like structures. This corresponded to similar von Kossa-positive areas in the matrix (arrows). M, vessel media. This corresponded to similar von Kosra-positive areas in the matrix (arrows). M, vessel media. **D**, Western blot showing small interfering RNA (siRNA) knockdown of specific annexins in VSMCs. **E** and **F**, Cells were transiently transfected with siRNAs targeting AnxA2, A5, or A6, followed by treatment with calcifying media (2.7 mmol/L calcium/2.5 mmol/L phosphate). Only knockdown of AnxA6 reduced Alizarin Red staining (**E**) and significantly decreased ^{45}Ca incorporation. n=6, $^{**}P<0.01$ (ANOVA).

and outer vesicle membranes. Although VSMC-MVs share some similarities with chondrocyte MVs, the absence of ALP activity and the abundance of Anx6 suggest they do not share all the properties of "professional" MVs released by hypertrophic chondrocytes, which are produced under regulated physiological conditions and primed for mineralization.[4] Rather, the shift to mineralization competency in VSMC-MVs is dependent on pathological changes in intracellular calcium homeostasis that disrupt inhibitor loading and cause membrane and annexin changes that favor hydroxyapatite nucleation. These mechanisms are more consistent with the dystrophic nature of human vascular calcification, which accumulates over a long time period and rarely manifests as true cartilage or bone.

Production of Mineralization-Competent Matrix Vesicles Is Regulated by Intracellular Calcium Homeostasis

In growth plate chondrocytes, retinoic acid is the physiological signal that induces an intracellular calcium rise that triggers the production of mineralization-competent MVs.[5,23]

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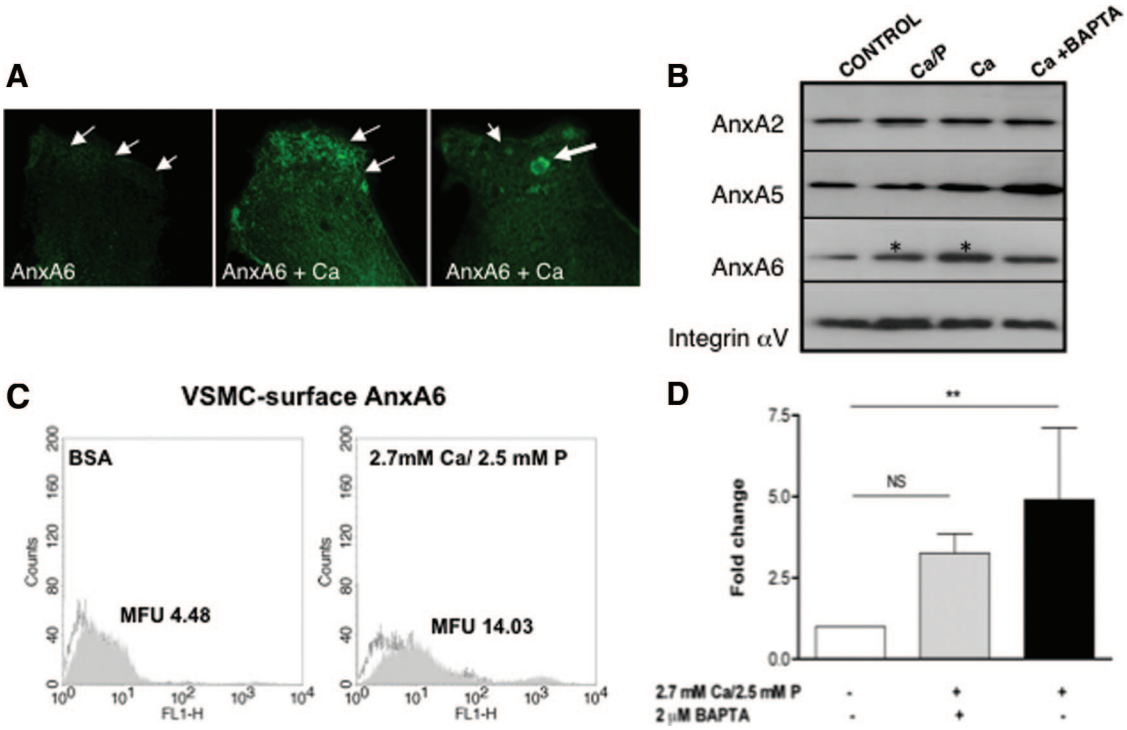

Figure 5. Calcium triggers changes in AnxA6 localization in VSMCs. A, Immunofluorescence showed that in control media, AnxA6 was cytosolic and only weakly associated with the plasma membrane (left). In response to calcifying media (Ca/P, 2.7 mmol/L calcium/ 2.0 mmol/L phosphate) for 6 hours, AnxA6 increased at the plasma membrane (**arrows**, middle) and localized to vesicular structures (**arrow**, right). Magnification ×1000. **B**, Biotin cross-linking confirmed that AnxA2, A5, and A6 were localized to the plasma membrane of VSMCs. In response to treatment with calcifying media for 3 hours, AnxA6 accumulated at the plasma membrane (**asterisks**), and this was attenuated in response to BAPTA-AM treatment. Plasma membrane–associated AnxA2 and A5 did not change. Integrin-αV is shown as a membrane control. **C**, Flow cytometry confirmed that AnxA6 was enriched at the plasma membrane of VSMCs in response to extracellular calcium, as shown by increased fluorescence. This enrichment was reduced by treatment with BAPTA-AM, shown graphically in panel **D** as mean fold change of mean fluorescent units (MFU). n=5, mean±SD. $^{**}P<0.01$.

The mechanisms involve upregulation of AnxA2, A5, and A6 and their incorporation into mineralizing MVs, in which AnxA5, the most abundant annexin, is key in mediating mineral nucleation.[5,6,24] In the present study, we also demonstrated a crucial role for cytosolic calcium homeostasis in the regulation of VSMC mineralization. Chelation of intracellular calcium blocked AnxA6 shuttling in response to extracellular calcium and MV calcification; however, in contrast to the physiological signaling observed in chondrocytes, pathological extracellular calcium stress induced VSMCs MV mineralization. Previous studies have suggested that MV release by VSMCs is an adaptive response aimed at preventing intracellular calcium overload.[25,26] The normal loading of MVs with inhibitors such as MGP and fetuin-A, as well as the ability of these inhibitors to suppress MV calcification, supports this idea. The specific loading of VSMC-MVs with AnxA6 is also consistent with this notion in light of recent data that suggest that AnxA6 is involved in the maintenance of intracellular calcium homeostasis and in limiting cellular damage due to calcium overload.

Studies have shown that the calcium-dependent translocation of AnxA6 to the plasma membrane occurs after an influx of extracellular calcium.[27–29] At the plasma membrane, AnxA6 functions as a membrane microdomain organizer and can regulate the activity of ion channels involved in both calcium entry and efflux in a cell- and context-specific manner.[30–33] For example, calcium-induced binding of AnxA6 to the plasma membrane stabilizes the actin cytoskeleton and efficiently prevents store-operated calcium entry in HEK293 cells, whereas AnxA6 knockout mice show accelerated calcium efflux during cardiomyocyte contraction.[30,31] AnxA6 translocation to the plasma membrane also preceded the sealing off of "hot spots" of extracellular calcium entry into cells and the subsequent shedding of microparticles, which led to a reduction in intracellular calcium and cell recovery.[29] The present data are consistent with the notion that under sustained conditions of calcium overload, AnxA6 shuttles to the plasma membrane, where it acts to regulate calcium homeostasis and vesicle release. We also showed that AnxA6 knockdown acted to reduce VSMC calcification. Potentially, the lack of AnxA6 on MV membranes may have reduced their capacity to nucleate hydroxyapatite. Alternatively, AnxA6 may have a specific role in calcium homeostasis in VSMCs. Thus, loss of AnxA6 could potentially impinge on multiple cellular processes, including calcium transport, proliferation, apoptosis, differentiation, or membrane dynamics, and therefore, further investigation of these mechanisms is required.[22]

Phosphatidylserine and Annexin Complexes Mediate Calcification

VSMC mineralization-competent MVs were also enriched with phosphatidylserine on the outer surface compared with nonmineralizing MVs, and externalization of phosphatidyl-

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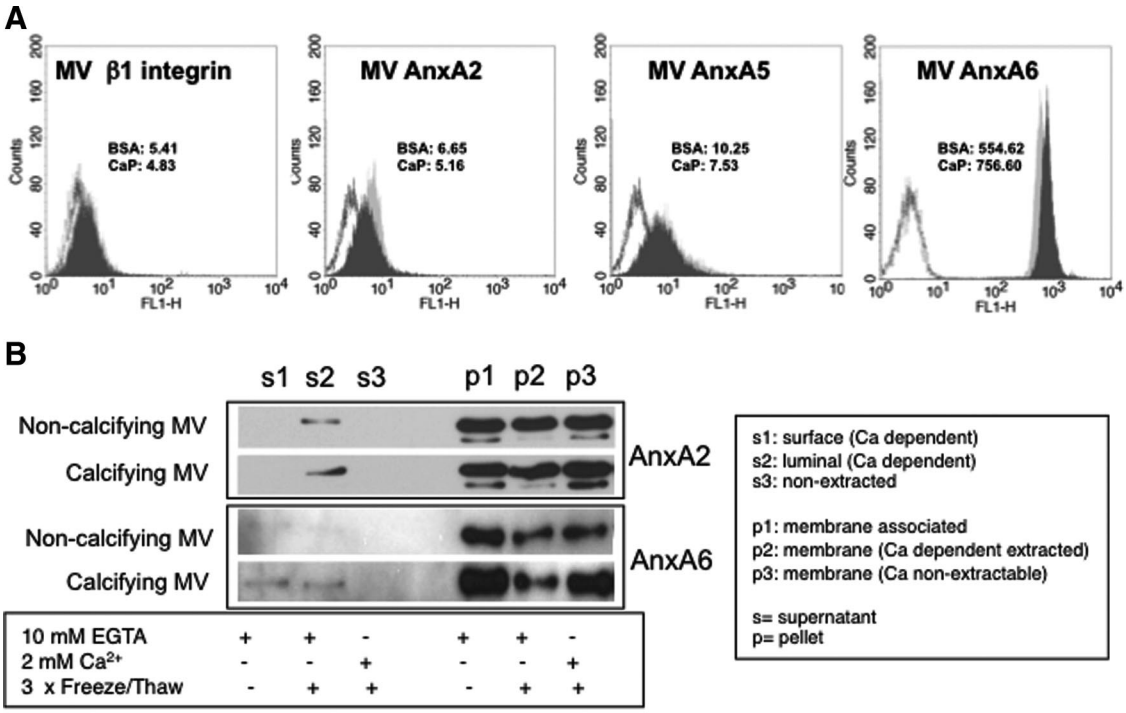

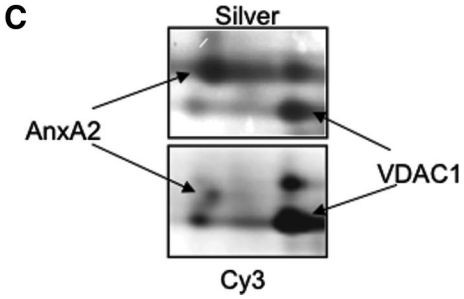

Figure 6. Surface and membrane-associated AnxA6 is enriched in MVs. A, Flow cytometric analysis of integrin b1 on MV membranes indicated they had the same orientation as the plasma membrane. Annexins were detectable on the MV membrane, but only AnxA6 was enriched in response to calcium/phosphate (2.7 mmol/L calcium/ 2.5 mmol/L phosphate) treatment (mean fluorescent units are shown). Representative data for n=4 independent experiments. **B**, AnxA6 was extracted by EGTA from both the outer (S1) and luminal (S2) surfaces of MV membranes, whereas AnxA2 was present only on the luminal membrane (s2). Note also the increase in membrane-bound AnxA6 in mineralization-competent MVs (p1 and p3). **C**, Two-dimensional difference gel electrophoresis analysis confirmed the luminal localization of AnxA2. AnxA2 was present only in the silver-stained gel and not in the Cy3 membrane-labeled protein fraction. In contrast, the membrane-bound calcium channel VDAC was most prominent in the Cy3-labeled membrane protein fraction.

serine was induced by raised intracellular calcium. Studies have shown that phosphatidylserine is also abundant on chondrocyte-derived MVs, where it forms hydroxyapatite nucleation complexes, predominantly with AnxA5.[6,34,35] In chondrocytes, phosphatidylserine exposure is induced by raised intracellular calcium, which stimulates the activity of a scramblase, which causes phosphatidylserine exposure, and this mechanism may also be active in VSMCs.[34] Although nonspecific calcium-dependent flip-flop of phospholipids has been reported in apoptotic cells, and "default" calcification of apoptotic bodies is also thought to be mediated by externalized phosphatidylserine, the majority of VSMCs that exposed surface phosphatidylserine were not apoptotic.[36,37]

Indeed, although apoptosis is a key event in VSMC calcification, the present data suggest that MVs differed significantly from apoptotic bodies with respect to size, annexin composition, and mineralization capacity, which suggests MVs have additional mechanisms to enable luminal mineralization.[12,16] Phosphatidylserine-annexin complexes may be sufficient to rapidly and efficiently nucleate hydroxyapatite without the requirement for annexin calcium channel activity[6]; however, proteomics revealed that VSMC-MVs contain additional proteins with possible roles in calcium and phosphate uptake. The plasma membrane enzyme 5′-nucleotidase hydrolyzes nucleotides to phosphate and their corresponding nucleosides, which provides an additional phosphate source.[8,38] The voltage-dependent, anion-selective channel protein 1 (VDAC1) has calcium channel activity,[39] although 2 additional proteins implicated in the regulation of cation channel activities, SLP-2 (stomatin-like protein 2) and calmodulin, were also identified.[40,41] Whether these proteins are functional remains to be tested. To date, only raised levels of extracellular calcium have been shown to induce the formation of mineralization-competent MVs by VSMCs. Thus, VSMC-MVs may acquire elements of the calcium-homeostasis machinery because of the calcium-dependent accumulation of these ion channels in AnxA6-rich membrane microdomains (rafts), which are subsequently shed.[27,29] Importantly, this mechanism is consistent with vascular mineralization occurring at sites of cellular damage in the media and intima, where cell death can induce microenvironments high in extracellular calcium.[10,26] However, it will be important to determine whether other calcifying stimuli such as hyperlipidemia, hyperglycemia, or inflammatory cytokines

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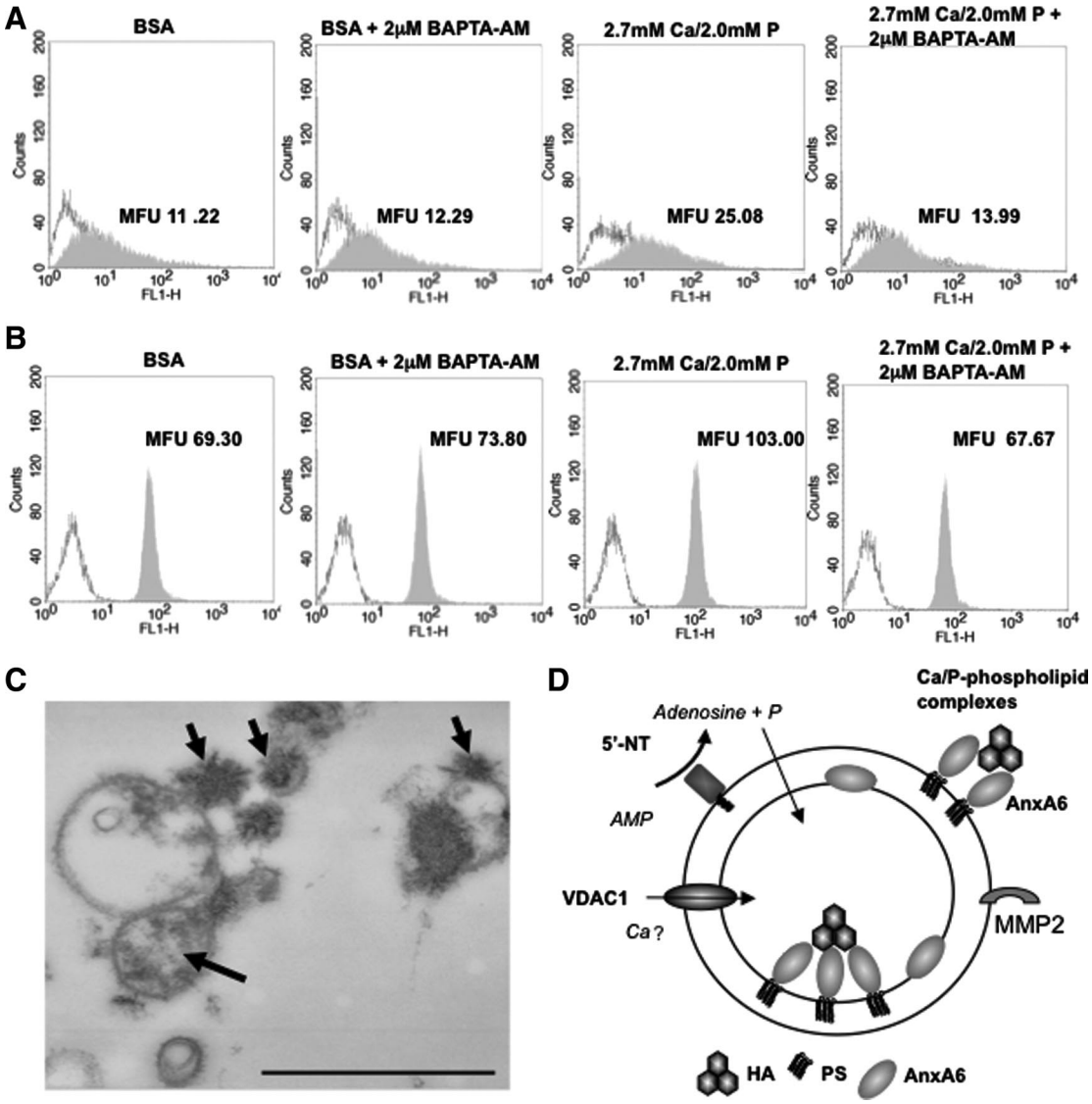

Figure 7. Phosphatidylserine is exposed on MVs in response to extracellular calcium. A, Flow cytometry showed that phosphatidylserine was exposed on the surface of VSMCs in response to calcifying media, and this was blocked by BAPTA-AM treatment. Data show mean fluorescence units (MFU) and are representative of n=4 independent experiments. Ca, calcium; P, phosphate. **B**, Calcium-dependent accumulation of phosphatidylserine on the MV outer surface was also induced by calcifying media. Data show MFUs and are representative of n=4 independent experiments. **C**, Transmission electron microscopic analysis of mineralization-competent MVs isolated from VSMCs showing MVs with crystalline hydroxyapatite associated with both the outer (**arrowheads**) and inner (**arrow**) membranes. **Scale** bar, 0.5 μm. **D**, Model showing potential mechanisms of MV calcification. Phosphate is released by hydrolysis of nucleotides catalyzed by 5′-nucleotidase (5′-NT) and taken up via phosphate transporters, whereas calcium conduction across the MV membrane is potentially mediated by VDAC1. Mineral ions, calcium and phosphate, bind AnxA6 and phosphatidylserine (PS) to form nucleation complexes on the outer and inner surfaces of MVs to promote crystal apatite formation. MMP2 on the MV surface acts to degrade elastin and promote calcification.

can induce MV release and mineralization, potentially by affecting intracellular calcium homeostasis.

Calcium Induces Loss of MGP, a Potent Inhibitor of MV Mineralization

Another crucial factor that led to increased production of mineralization-competent MVs by VSMCs in response to calcium was the reduction in MGP protein loading. In chondrocytes, MGP is present in nonmineralizing MVs but is absent in mineralization-competent MVs.[42,43] Similarly, in the normal vessel wall, VSMC-MVs were noncalcified, which suggests they contained inhibitors; however, this inhibition is apparently lost in the diseased vessel wall.[10] Downregulation of MGP loading into MVs could be due to exhaustion of the protein because of prolonged calcium overload or impairment of intracellular sorting mechanisms. Importantly, accumulation of the uncarboxylated form of MGP occurs at sites of calcification, and MGP posttranslational modifications occur in the endoplasmic reticulum, an organelle likely to be impaired by calcium overload.[10]

Origin and Alternate Functions for VSMC-MVs

The observation that VSMCs release MVs under noncalcifying conditions suggests that these particles may serve a

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functional role in the cell beyond pathological calcification. Proteomics analysis revealed a number of ECM components, as well as integrins, which suggests MVs may be involved in the formation of ECM and may also be targeted for integrin-dependent ECM binding. MVs also exhibited MMP2 activity, and freeze/thaw analysis (data not shown) indicated that this activity was most likely on the MV surface. MVs were found in tight association with collagen and elastin fibers, and under normal conditions, MMP2 activity may be required during matrix biogenesis. However, elastin degradation also promotes calcification, and the activation of MV MMP2 by calcium suggests this may lead to enhanced degradation, as well as the production of elastin peptides, both of which are key inducers of calcification.[20,44] Signaling molecules, chaperones, and proinflammatory factors such as cyclophilin A were also detected, which suggests that MVs may act as intercellular signaling modules, similar to the role described for exosomes in other cell types.[45] Although the presence of cell-surface markers on MVs and actin and actin regulatory proteins (moesin, cofilin, WD repeat-containing protein 1, F-actin–capping protein, and tropomyosin) is consistent with a plasma membrane origin, other proteins involved in vesicle biogenesis were also identified, which may point to an endosomal origin for some vesicles.[46] Clearly, further studies are now required to characterize these VSMC-derived organelles more fully under normal and calcifying conditions.

Acknowledgment

We acknowledge Dr Slawomir Pikula from the Nencki Institute of Experimental Biology for protocols and helpful discussion. We also acknowledge Dr Xiaoke Yin for excellent technical support.

Sources of Funding

This work was supported by a British Heart Foundation program grant to Catherine M. Shanahan (RG/05/001).

Disclosures

None.

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Novelty and Significance

What Is Known

- Vascular smooth muscle cells (VSMCs) are known to be key participants in the initiation of vascular calcification in both the intima and media of the vessel wall.
- Dysregulated mineral metabolism leading to increases in calcium and phosphate levels promotes VSMC calcification.
- Matrix vesicles released by VSMCs are found at sites of calcification, but the mechanisms whereby these structures promote calcification in response to changes in calcium and phosphate levels are poorly understood.

What New Information Does This Article Contribute?

- Calcification is initiated primarily in matrix vesicles rather than directly on the extracellular matrix.
- Dysregulation of intracellular calcium plays an important role in initiating matrix vesicle calcification.
- Elevated levels of extracellular calcium initiate matrix vesicle calcification by interfering with inhibitor loading and by promoting the formation of hydroxyapatite nucleation complexes.

Vascular calcification is a detrimental process associated with increased cardiovascular mortality. In association with dysregulated mineral metabolism, it is particularly prevalent in chronic kidney disease. VSMCs mediate the calcification process in response to elevated levels of extracellular calcium and phosphate. Although the mechanisms by which elevated phosphate induces calcification are well studied, the role of elevated calcium is less well understood. In the present study, we show that elevated calcium promotes calcification of matrix vesicles, small membrane-bound structures that are released by VSMCs that form the first nidus for calcification in the vessel wall. Elevated calcium triggers VSMC calcification by altering intracellular calcium levels. This calcium overload induces the production of specialized matrix vesicles that are enriched with annexins and membrane lipids. These vesicles are able to initiate calcification because they lack important calcification inhibitors. Importantly, maintenance of normal intracellular calcium levels prevents these events. Clinically, this suggests that the maintenance of plasma calcium levels in the normal range and reductions in bouts of transient hypercalcemia are important for the prevention of vascular calcification, particularly in patients with chronic kidney disease.

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SUPPLEMENTAL MATERIAL

Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization

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Online Supplement Materials and Methods

Tissue Samples

Human aortic samples were obtained with appropriate ethical approval from transplant donors and recipients and carotid endarterectomy surgery. Explant cultures of human VSMCs were established from human aortic tissue from both male and female organ donors of various ages as previously described[1]. Cells were cultured in M199 (Sigma) supplemented with 20% FBS and antibiotics at 37°C in a 5% CO_2 incubator and used between passages 3 and 12.

Induction and Analysis of VSMCs Calcification

VSMCs were grown to 80% confluence, then washed three times with Earle's Balanced Salt Solution (EBSS) and incubated in control media (M199 supplemented with 0.5% BSA and containing 1.8 mmol/L $CaCl_2$/1.0 mmol/L NaH_2PO_4) or calcifying media (M199 supplemented with 0.5% BSA and containing either 5.4 mmol/L $CaCl_2$ / 1.0 mmol/L NaH_2PO_4 or 2.7 mmol/L $CaCl_2$/2.5 mmol/L NaH_2PO_4 unless otherwise indicated) and ^{45}Ca (~50,000 cpm/mL). These concentrations of extracellular mineral ions have previously been shown to induce the release of mineralization competent MV from VSMCs[2]. Media with raised levels of extracellular Ca (3.6 mmol/L) or P (2.5 mmol/L) alone were used for comparison in some experiments as stated. After incubation (approximately 16-24 hours) VSMCs were decalcified in 0.1mol/L HCL, neutralized by the addition of 0.1 mol/L NaOH/0.1% SDS, scraped and ^{45}Ca incorporation was measured by liquid scintillation counting. Experiments were performed in triplicate and independently verified in at least 3 different VSMC isolates. For experiments examining cytosolic calcium regulation 1-5 μmol/L BAPTA-AM (Invitrogen, Eugene, OR) was added at the time of calcium addition. BAPTA-AM is an intracellular Ca chelator that has no effect on extracellular Ca levels but can chelate intracellular Ca within the micromilar range. K201 (Aetas Pharma) was added at concentrations between 1 μm- 10 μm at time of calcium addition. Cell calcification was also visualized by staining with alizarin red (2% aqueous, Sigma) as described previously[1].

To examine the role of MMP2, VSMCs were treated as above in the presence of the MMP inhibitor GM6001 (Calbiochem) for 16 hours. Then Ca content in

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VSMCs was measured colorimetrically using a modified o-cresolphthalein complexone method[3]. In brief, VSMCs were washed by HBSS, incubated in 0.1 mol/L HCl for 24 hours at 4°C and spun down by centrifugation at 16,100 x g for 10 minutes. Supernatants were mixed with o-cresolphthalein complexone reagent (Sigma, St Louis, MO) and absorbance at 540 nm was measured using a spectrophotometer (Tecan GENios Pro). Ca concentration was calculated using the standard curve obtained with Ca standard solution. The final Ca content in the VSMCs was standardized for the protein content.

Protein concentration

Protein concentration was determined by DC protein assay (BioRad).

Preparation of VSMC Matrix Vesicles and Apoptotic Bodies

AB were isolated from Fas-ligand treated VSMCs as previously described[1]. VSMC MV were harvested by differential centrifugation as described earlier[4]. Briefly, VSMCs cultures were grown until 90% confluent, washed twice with EBSS and transferred to control media (DMEM supplemented with 0,1% BSA) or calcifying media (DMEM containing 0.1% BSA and calcium and/or phosphate as indicated). $CaCl_2$ and NaH_2PO_4 were added to supplement calcium and phosphate in the media. Cell media were collected after approximately 16 hours and then subjected to centrifugation at 2500 rpm (Thermo Scientific Heraeus Multifuge 3SR+ centrifuge, rotor Sorvall 75006441K) for 5 min to remove AB and larger vesicles. MV were harvested from the media by centrifugation at 35,000 rpm (100,000 x g) for 40 minutes at 4°C (Beckman Coulter Optima Max Unltracentrifuge). MV were resuspended in PBS (Sigma) and protein concentration was determined.

^{45}Ca Uptake by VSMCs Matrix Vesicles

MV calcification assays *in vitro* were performed using modified method of Hashimoto[5]. Briefly, 5 µg of MV were incubated in calcifying 50 mmol/L TES buffer (pH 7.6) containing 2.2 mmol/L $CaCl_2$, 1.6 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, 85 mmol/L NaCl, 15 mmol/L KCl, 10 mmol/L $NaHCO_3$ and ^{45}Ca (50,000 cpm/ml) at 37°C for 24 hours and spun down by centrifugation at 13,000 rpm for 10 minutes. Pellets were washed with HBSS, spun down by centrifugation at 13,000 rpm

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for 10 minutes and resuspended in 0.1 mol/L HCL. Then samples were neutralised with 0.1% SDS/0.1 mol/L NaOH and incorporated ^{45}Ca was measured by liquid scintillation counting. K201 (1-100 μm) and antibodies to AnxA2 and AnxA6 and IgG control (0.1 μg/100 μl) were added with the calcifying buffer solution.

Transmission Electron microscopy (TEM)

Transmission Electron Microscopy was performed as previously described on human samples obtained from normal controls and patients with diabetic peripheral vascular disease (femoral artery) or on dialysis[6]. Vessels were fixed in 4% glutaraldehyde containing 2 mmol/L $CaCl_2$ in 0.1 mol/L PIPES buffer (pH 7.4), supplemented with 0,33% H_2O_2 immediately before use. Samples were incubated for 4 hours at 4°C, washed twice in 0.1 mol/L PIPES (pH 7.4) and kept at 4°C. After the post-fixation in 1% osmium ferricyanide for 1 hour samples were rinsed 3 times in DIW and incubated in 2% uranyl acetate for 1 hour. Then samples were rinsed in water and dehydrated in an ascending series of ethanol solutions to 100% ethanol, rinsed twice in acetonitrile and embedded in Quetol epoxy resin (9.0 g Quetol 651, 11.6 g nonenylsuccinic anhydride (NSA), 5.0 g methylnadic anhydride (MNA) and 0.5 g benzyl dimethylamine). Tissue sections (50 nm) were cut on using a Leica Ultracut UCT and stained with saturated uranyl acetate in 50% ethanol and lead citrate. Samples were analysed using FEI Philips CM100 (80kv).

Tissue immunohistochemistry.

Immunohistochemistry was performed on human carotid endarterectomy (N=6) and normal and calcified human aortic samples (N=4) as previously described[6]. Antibodies used were annexin A2 (BD Transduction Laboratories 1:500), annexin A5 (Abcam; 1:500) and annexin A6 (BD Bioscience; 1:500). Tissue calcification was revealed using von Kossa staining. VSMCs and macrophages were identified using antibodies to α-SM actin and CD68 respectively.

Western Blotting

VSMCs were washed once and scraped in PBS, pelleted at 700 x g for 5 min (Thermo Scientific Heraeus Multifuge 3SR+) and lysed in RIPA buffer containing, 100 mmol/L Na_3VO_4, 20 μl/ml phosphatase inhibitor cocktail (1:50, Sigma) and

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protease inhibitor cocktail (1:50, Sigma) or 0,1 mol/L Tris buffer (pH 8.1), containing 0.15 mol/L NaCl, 1% triton X-100 and protease inhibitor cocktail (1:50). Lysates were subjected to short sonication (5 s, setting level 1, Branson Sonifier 150) and centrifuged at 13,200 x g for 15 minutes (Scientific Heraeus Multifuge 3SR+ centrifuge). Supernatants were collected and stored at -20°C. Protein concentration of lysates was determined using DC protein assay (Bio-Rad). Samples (5 µg) were separated on 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore) using semi-dry transfer cell (Bio-Rad). The membrane was incubated in blocking buffer (PBS containing 5% dry milk and 0.05% Tween-20) and then incubated with primary antibody. Antibodies used for detection were annexin A2 (BD Transduction Laboratories), annexin A5 (Abcam), annexin A6 (BD Bioscience), vinculin (Sigma), MGP (A monoclonal antibody raised against aa 3-15, 1:250). After extensive washing in blocking buffer the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (General Electric, 1:5000) diluted in blocking buffer, washed in PBS containing 0.05% tween-20 and visualized using the ECL or ECL+ system (Amersham). Coomassie Blue staining (BioRad) was used to demonstrate equal sample loading. Developed films were scanned using a scanner (Epson Perfection 2400) and image band intensity was measured using Scion Image 4.0.3.2 software.

Alkaline Phosphatase Assays

VSMCs or MV was lysed in 10% SDS on ice and centrifuged at 13,200 x g for 5 min (Thermo Scientific Heraeus Multifuge 3SR+). Supernatants were collected and ALK activity was measured spectrophotometrically at 405 nm using a p-nitrophenyl phosphate substrate (Sigma). Protein concentration was determined in lysates using DC protein assay (BioRad) and relative ALP activity was calculated and normalized to protein content.

Analysis of MMP Activity by Gelatin Zymography

Gelatin zymography was performed using the method of Yang et al[7]. In brief, MV (5 µg) were mixed with sample buffer (62.5 mmol/L Tris (pH 7.4), 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromphenol blue) and separated in 10% SDS-PAGE containing 1 mg/ml gelatine by running at 40 mA for 90 min. To remove SDS

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gels were washed in 2.5% Triton X-100 for 15 min twice and then incubated in 50 mmol/L Tris-HCl (pH 7.4) buffer containing 10 mmol/L $CaCl_2$ and 0.05% Brij 35 overnight at 37°C. Then gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250, 10% (v/v) glacial acetic acid, and 30% (v/v) methanol and the gelatinolytic activity of MMP2 (72 kDa gelatinase) were revealed by distaining with 10% (v/v) acetic acid and 30% (v/v) methanol.

MMP2 activity

MMP2 activity was measured using SensoLyte520 MM2 Assay Kit (AnaSpec, Fremont, CA) according to the manufacturer's protocol. Briefly, isolated MV or AB (2.5 µg) were re-suspended in the assay buffer containing 0.1% Triton-X and centrifuged at 10000 x g for 15 min at 4°C. Supernatant was incubated with 1 mmol/L 4-aminophenylmercuric acetate (APMA) for 1 hour at 37°C and immediately mixed with 5-FAM/QXL™520 FRET substrate. Fluorescence intensity was continuously recorded at microplate reader (Tecan GENios Pro) with a filter set of Ex/ Em=485 nm/535 nm at 37°C and relative fluorescence units (RFU) were converted to the concentration of the product using fluorescence reference standard curve obtained with 5-FAM-Pro-Leu-OH fluorescence reference standard.

Cathepsin S activity.

Cathepsin S activity was measured using Ac-Lys-Gln-Lys-Leu-Arg-AMC substrate (AnaSpec, Fremont, CA) as described in[8]. In brief, 1.25 µg of MV or AB were incubated in 50 mmol/L sodium citrate buffer (pH 5.5) containing 4 mmol/L DTT, and mixed with 10 µmol/L Cathepsin S substrate. Fluorescence intensity were measured at microplate reader (Tecan GENios Pro) with a filter set of Ex/ Em= 340 nm/535 nm at 25°C over 1 hour.

Immunofluorescence

VSMCs were plated onto coverslips in the 24 well plate at a density 1.2 x 10^5 cells/well and grown for 48 hours. Cells were treated as indicated and fixed in 3.7% paraformaldehyde for 15 min at room temperature, washed and permeabilized with 0,1% triton X-100 for 5 min. VSMC were incubated in blocking buffer (PBS containing 3% BSA) and stained with primary antibodies as indicated. Then samples

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were incubated with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen) and examined by fluorescence microscopy using a Leica microscope.

Proteomic analysis of VSMCs MV by mass spectrometry

For one-dimensional gel electrophoresis (1D) and mass spectrometry analysis VSMCs MV were resuspended in SDS-PAGE denaturing buffer and heated to 90°C for 3 minutes. Proteins were separated on precast 4-15% gradient SDS-PAGE gel (Biorad), stained with Colloidal Blue (Invitrogen) and protein bands were excised. Excised gel pieces were destained, the proteins reduced by DTT and then alkylated by iodoacetamide to prevent disulphide bond formation. The gel was dehydrated and trypsin drawn into the gel by rehydration in an aqueous buffer. Digestion was run overnight, and the resulting peptides were retrieved from the buffer and analysed by LC-MS/MS. Mass spectrometry data were analyzed using Mascot program.

Two-dimensional Difference Gel Electrophoresis (2D-DIGE) was performed as previously described with modifications[9]. In brief, matrix vesicles (100 µg) were resuspended in labelling buffer, containing 1M urea/HBSS, pH 8.5 and incubated with Cy3 DIGE Fluor minimal dye (GE Healthcare, Little Chalfont, United Kingdom) with a dye/protein ratio of 1 nmol/100 µg, for 20 min on ice in the dark. To quench any remaining free Cy3, an equal volume of 10 mmol/L lysine was added to the reaction mix and incubated for 15 min at room temperature. The sample was mixed with the same volume of DIGE 2x buffer (8 mol/L urea, 0,5% (w/v) CHAPS, 0,2% (w/v)DTT and 2% (v/v) Pharmalytes pH 3-10), homogenized by ultrasound treatment and cleared by centrifugation at 800 x g for 5 min. The supernatant was loaded onto an IPG strip (18 cm, pH 3-10, nonlinear, GE Healthcare) and focused at 60 kV-h at 20°C with Multiphor II (GE Healthcare). Proteins were separated in the second dimension using an acrylamide gradient gel (4-12%) with the 2DEoptimizer with a standard Laemmli Tris/Glycine reagent pack (NextGen Science). After overnight fixation in methanol:acetic acid: water solution (4:5:1), Cy3 stained proteins were visualised on a Typhoon imager (GE healthcare) using the red (533 nm) laser and 670 nm emission filter. Total proteins were silver stained using Plus One silver stain kit (Amersham) with modification to ensure compatibility with further MS/MS analysis. Silver stained gels were scanned in transmission scan mode using a calibrated scanner (GS-800, BioRad). Detailed protocols for 2D-DIGE and silver staining are provided

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on-line (http://www.vascular-proteomics.com). Protein localization (inside/outside) was indicated based on the difference between Cy3 and silver staining for each protein spot. Protein spots differentially exhibited on Cy3 compared with Silver stained gels were excised and identified using LC-MS/MS. Proteins, obtained from the gel spots which were intensively stained on the Cy3 gel when compared with the Silver gel were marked as proteins presumably located on or associated with the outer membrane surface of MV ("out"). Proteins obtained from spots which were more strong stained with silver but not visible on the Cy3 Gel were marked as proteins presumably located inside the lumen of MV ("in"). The protein identification probability was set above 99%.

Biotin Crosslinking

VSMC surface labeling was performed using a Pierce Cell Surface Protein Isolation Kit (Pierce) according to the manufacturer's protocol. In brief, approximately 4×10^7 treated VSMCs were briefly rinsed with ice-cold PBS and biotinylated with membrane-impermeable EZ-Link Sulfo-NHS-SS-Biotin for 30 minutes at 4°C. The reaction was stopped by Quenching Solution and cells were lysed in Lysis Buffer and incubated for 30 min on ice. Cell lysates were centrifuged at 10,000 × g for 2 minutes at 4°C and biotinylated proteins were isolated from the supernatant using NeutrAvidin Agarose. Bound proteins were eluted in Laemmli Sample buffer containing 50 mmol/L DTT, separated by 10% SDS-PAGE and analyzed by Western blot.

siRNA transfection

Short interfering RNA (siRNA) oligonucleotides targeting annexins were pre designed by Qiagen and were: AllStars Negative Control siRNA; Hs_ANXA5_1 FlexiTube siRNA (SI00296975), Hs_ANXA2_10 FlexiTube siRNA (SI03060855), Hs_ANXA6_1 FlexiTube siRNA (SI00297003). Transient transfection of VSMC was performed using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer protocol. Briefly, cells were plated onto 48 well plate at a density $0.5*10^3$ cells/well and grown for 24 hours. siRNA was diluted in OptiMEM, mixed with HiPerFect Transfection Reagent and incubated for 10 min at room temperature. The complexes were added drop-wise onto the cells and VSMC were incubated 24 hours in M199 supplemented with 20% FBS and antibiotics. After the washing free

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times with EBSS, cells were either lysed in 0,1 mol/L Tris buffer (pH 8.1), containing 0.15 mol/L NaCl, 1% triton X-100 and protease inhibitor cocktail (1:50) and analyzed by western blotting to determine knockdown efficiency or incubated in serum-free M199 containing 0,5% BSA and antibiotics for 16 hours. Then cell were incubated in control media (M199 supplemented with 0,5% BSA, 1 µmol/L fetuin-A and containing 1.8 mmol/L $CaCl_2$/1.0 mmol/L NaH_2PO_4) or calcifying media (M199 supplemented with 0,5% BSA, 1 µmol/L fetuin-A and containing 2.7 mmol/L $CaCl_2$/2.5 mmol/L NaH_2PO_4) for 72 hours. Calcification of cells was examined by ^{45}Ca uptake or Alizarin Red staining.

Flow cytometry analysis of VSMCs

VSMCs were rinsed with EBSS twice, treated with trypsin and centrifuged at 700 x g for 5 min. Then cells were re-suspended in HBSS supplemented with 5% FBS and 1 x 10^5 cells were incubated with primary antibody or corresponding isotype-matched IgG controls for 30 min at +4°C. After washing twice with HBSS supplemented with 5% FBS cells were incubated with secondary antibody for 30 min at +4°, washed twice with HBSS supplemented with 5% FBS, and analysed with BD FACScalibur (BD Bioscience). Primary and secondary antibodies used in the study were phosphatidylserine antibody (Abcam), annexin A2 (BD Transduction Laboratories), annexin A5 (Abcam), annexin A6 (BD Bioscience), antibody for extracellular domain of integrin β_1 (Santa Cruz Biotechnology), Alexa Fluor 488 goat anti-mouse antibody (Invitrogen).

Analysis of matrix vesicles by flow cytometry

Matrix vesicles were coupled to 4 µm surfactant-free aldehyde/sulfate latex beads (Invitrogen) as described[10]. In brief, 5 µl of beads were mixed with 10 µg of MV in the PBS (final volume 100 µl) and incubated for 15 min at room temperature. The reaction volume was adjusted to 1 ml with PBS and the mixture was incubated for 16 h at 4°C on a rotator wheel plate. Free binding sites were blocked by the addition and incubation with 100 mmol/L glycine for 30 min at room temperature. Beads were collected by centrifugation at 4,000 x g for 3 min at 4 °C, and then resuspended and washed twice in 400 µl of PBS supplemented with 0,5% BSA. An aliquot of beads (20 µl) was incubated with primary antibody (final volume 90 µl),

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washed twice with PBS supplemented with 0,5% BSA, and incubated with secondary antibody in the final volume 50 µl. Beads were washed twice with PBS supplemented with 0,5% BSA and analysed by flow cytometry on BD FACScalibur (BD Bioscience). Single beads were gated to analyze fluorescence from the dot blot representation of forward (FSC) and side scatter (SSC) using Cell Quest Software. Mean fluorescent units (MFU) of the histogram were used to determine fold change. MFU of isotype-control stained beads was subtracted from the specific MFU value and the ratio between treated and a control cells was calculated.

Freeze/thaw analysis of MV

An aliquot of VSMC MV (5 µg) was resuspended in 50 mmol/L TES buffer (pH 7.6) containing 2.2 mmol/L $CaCl_2$, 1.6 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, 85 mmol/L NaCl, 15 mmol/L KCl, 10 mmol/L $NaHCO_3$ and 10 mmol/L EGTA was added. Samples were incubated for 15 min at room temperature and then MV and the fraction containing extracted outer membrane compounds were separated by ultracentrifugation at 100,000 x g for 30 min. Pelleted MV were resuspended in control TES buffer or test TES buffer containing 10 mmol/L EGTA only or 10 mmol/L EGTA and 2 mmol/L $CaCl_2$. Samples were disrupted by freezing/thawing in liquid nitrogen for 4 times. MV were isolated from luminal extracted proteins by centrifugation at 100,000 x g for 30 min and resuspended in TES buffer. Collected fractions were lysed in SDS-PAGE denaturing buffer, heated at 95°C for 5 min and analysed by western blot.

Statistics

Data were analysed by one-way ANOVA test with Bonferroni post test using PRISM software (GraphPad, San Diego, CA) or t-test as appropriate. Values of $p < 0.05$ were considered statistically significant.

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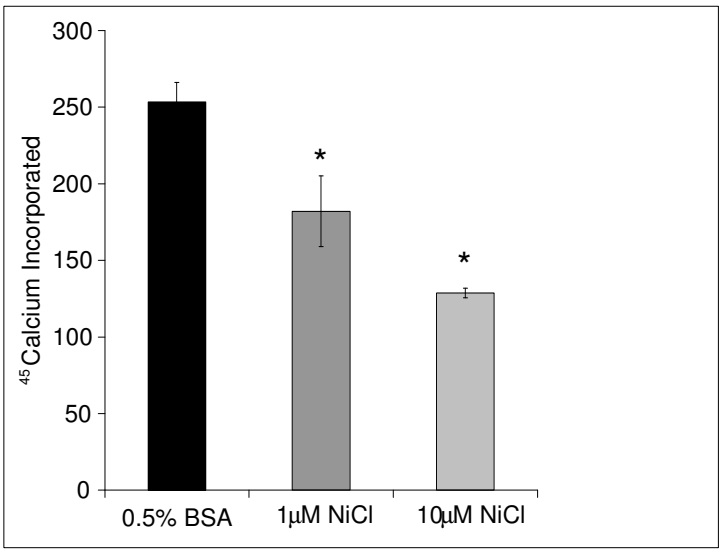

Figure I. VSMCs were treated with the Ca-uptake inhibitor NiCl in the presence of control (1.8 mM) or calcifying media containing 2x Ca (3.6 mM). ^{45}Ca incorporation was measured after 6 hours. The inhibition of Ca-uptake by VSMCs significantly inhibited calcification. *$p<0.05$, n=3. ANOVA

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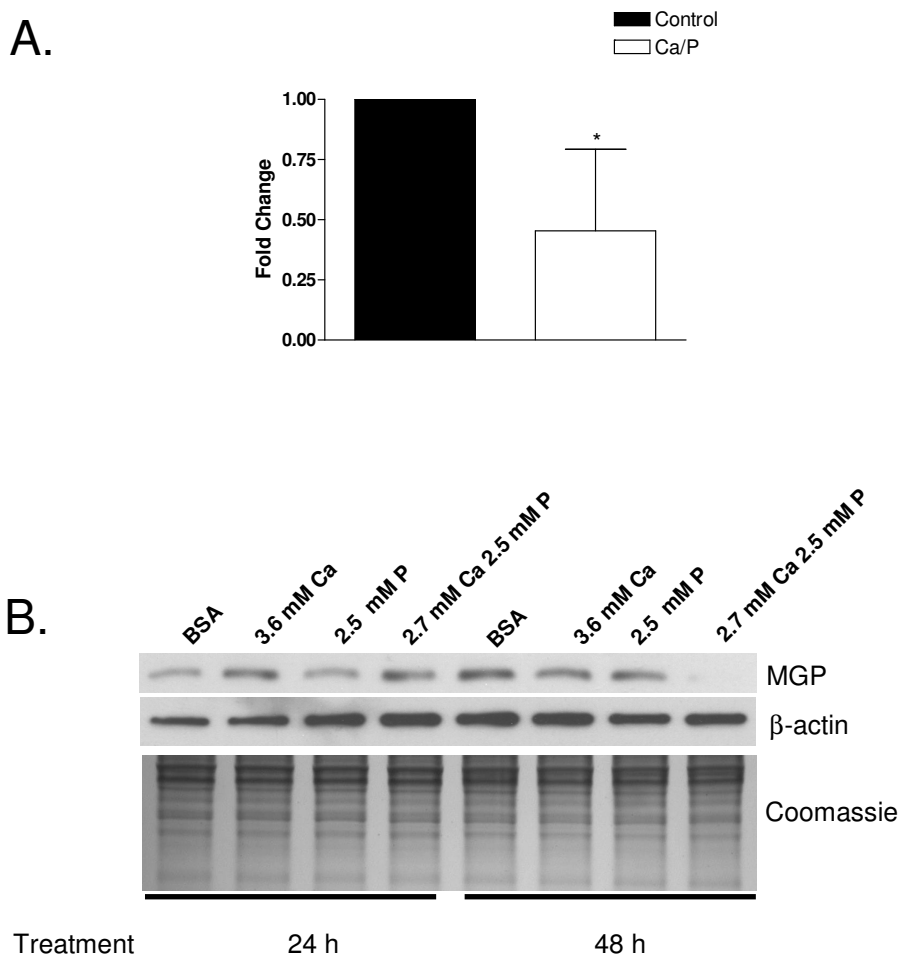

Figure II. MGP expression is altered in calcifying conditions.
A. Quantification of Western Blots of MGP protein in MV treated with Ca/P (2.7 mM $CaCl_2$/ 2.5 mM NaH_2PO_4) media for 48 hours. N=4, *$p<0.05$, Students t-test.
B. Western blot showing MGP expression in VSMC treated in calcifying conditions as indicated for 24 and 48 hours. Note the increase in MGP expression in response to Ca at 24 hours and its decrease or complete absence after 48 hours treatment. Treatment with P did not change MGP expression at any time point.

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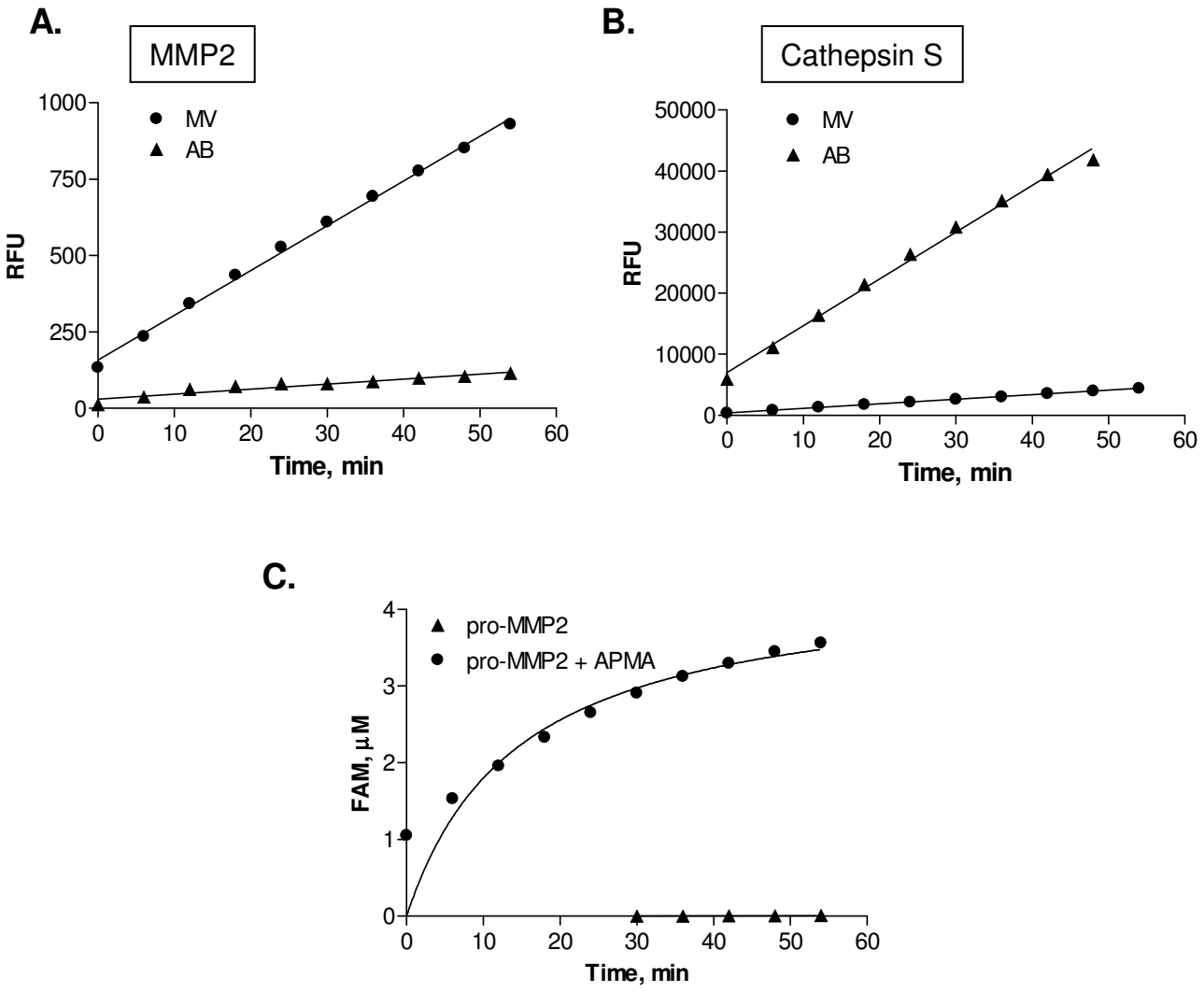

Figure III. VSMC calcification is mediated by MV-associated MMP2. A. Fluorimetric kinetic analysis of MMP2 activity shows enrichment of MMP2 activity in MV over AB. MMP2 activity was measured using MMP2 FRET substrate. Fluorescence intensity was continuously recorded at Ex/ Em=485 nm/535 nm. RFU, relative fluorescence units. **B.** Kinetic analysis of Cathepsin S activity in MV and AB shows higher activity of Cathepsin S in AB. Cathepsin S activity was measured using Cathepsin S substrate fluorescently labeled with AMC. C. Kinetic analysis shows activation of pro-MMP in the presence of APMA. Recombinant human pro-MMP (10 ng) was incubated in the assay buffer in the presence or absence of 1 mM APMA for 1 hour at 37℃ and MMP2 activity was measured using MMP2 FRET substrate. FAM = 5-FAM, 5-carboxyfluorescein.

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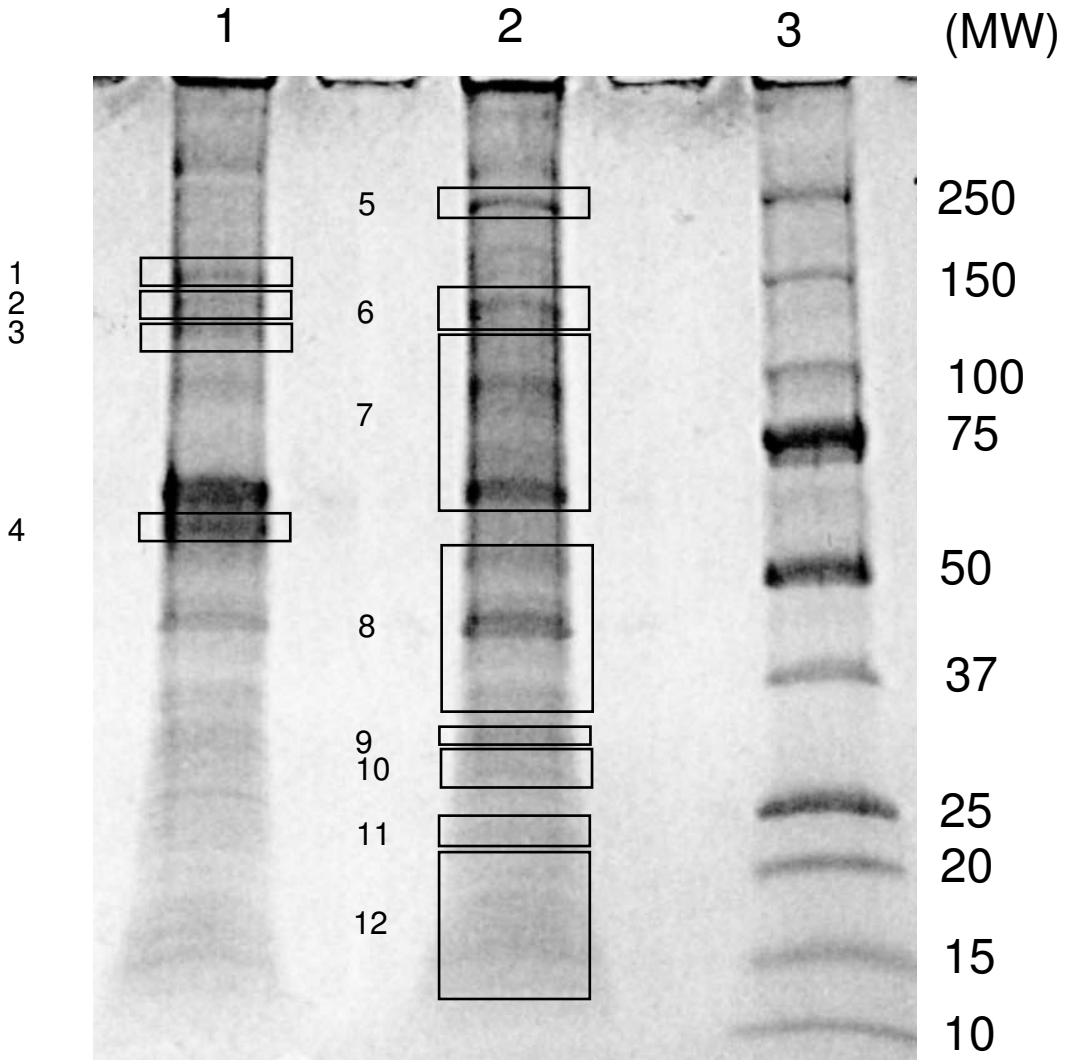

Figure IV. Separation of MVs proteins by 1D SDS-PAGE.

MV isolated by ultracentrifugation from media of VSMCs treated in the control media (Lane 1) or calcifying media containing 2.7mM $CaCl_2$/2.0 mM NaH_2PO_4 (Lane 2), were run on 4-15% gradient gel. Gel were stained with a colloidal blue staining kit, gel slices were cut as annotated, trypsin digested and analyzed by LC-MS/MS. Boxed areas show regions where differential expression of protein bands was detected. The full list of identified proteins is provided on Supplementary Table 1. Figure shows representative gels. Lane 3, molecular weight (MW) markers, kDa.

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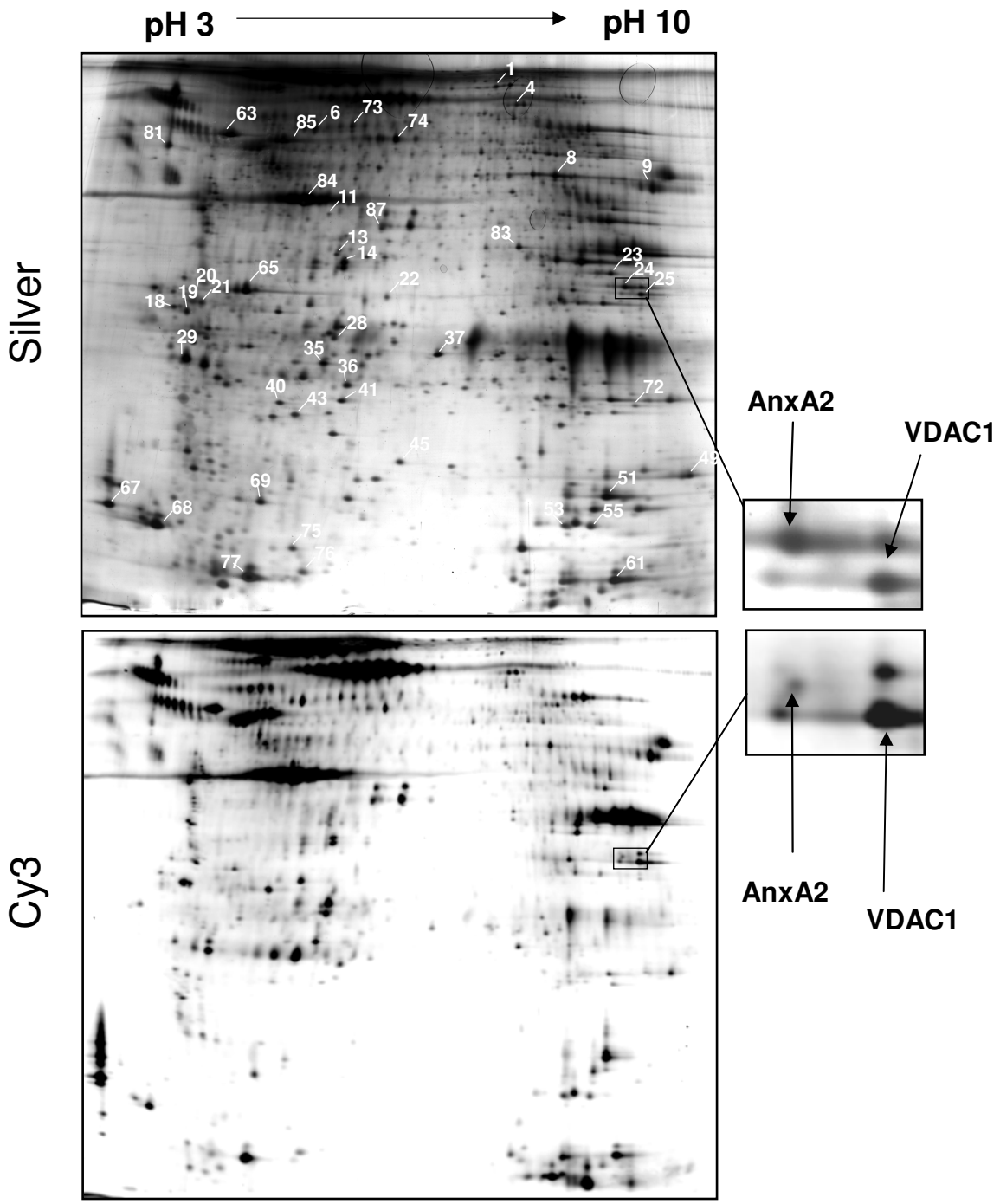

Figure V. Identification of membrane proteins in VSMC-derived MV using surface labelling and 2D-DIGE.

To examine membrane protein composition MV were labelled with Cy3 in non-permeabilizing conditions followed by separation by 2D-DIGE gel electrophoresis and silver stained. Fluorescence and silver stained images were acquired and the numbered spots that differentially appeared on the silver stained gel and the Cy3 visualized image were excised and identified by LC-MS/MS (See Supplementary Table 2 for a complete list of proteins).

Box – enlarged image of the gels showing that membrane protein VDAC1 is labelled with Cy3 dye and Silver staining whereas AnxA2 is detected mainly on the silver stained gel implying it is not abundant on the surface of MV.

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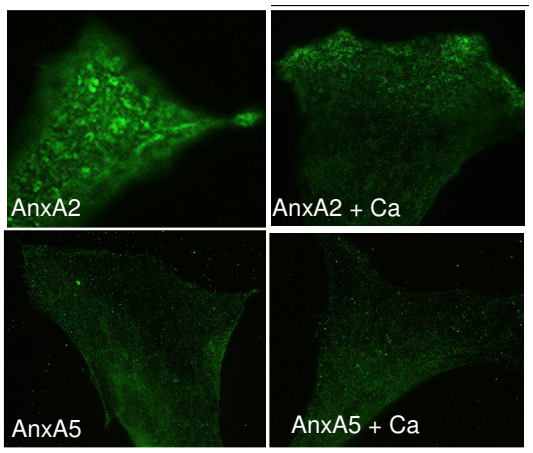

Figure VI. Immunofluorescence showing the presence of AnxA2 at the plasma membrane of VSMCs in control media and relatively little change in localization in response to Ca/P (2.7 mM $CaCl_2$/2.0 mM NaH_2PO_4) media for 6 hours. AnxA5 is cytosolic in control media and remains so in response to Ca/P media. Magnification, 1000X.

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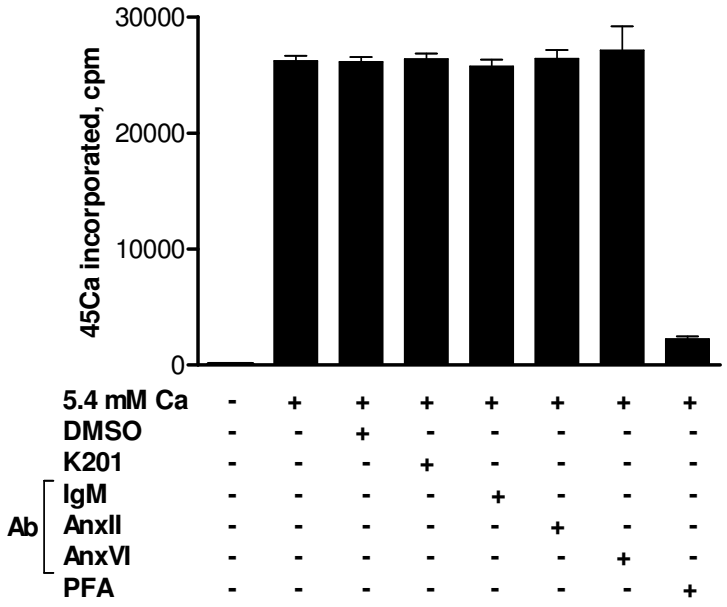

Figure VII. ^{45}Ca uptake by isolated VSMC-derived MV was not be inhibited by the selective annexin Ca-channel inhibitor, K201 or annexin antibodies. Phosphoformic acid (PFA) was used as a control inhibitor as this binds to HA and blocks crystal formation. Data show that MV treated with calcifying media were mineralization competent however calcification was not inhibited by blocking annexin channel activity.
Data are mean ± SD, n=3. Similar results were obtained in the presence of Ca/P calcifying media.

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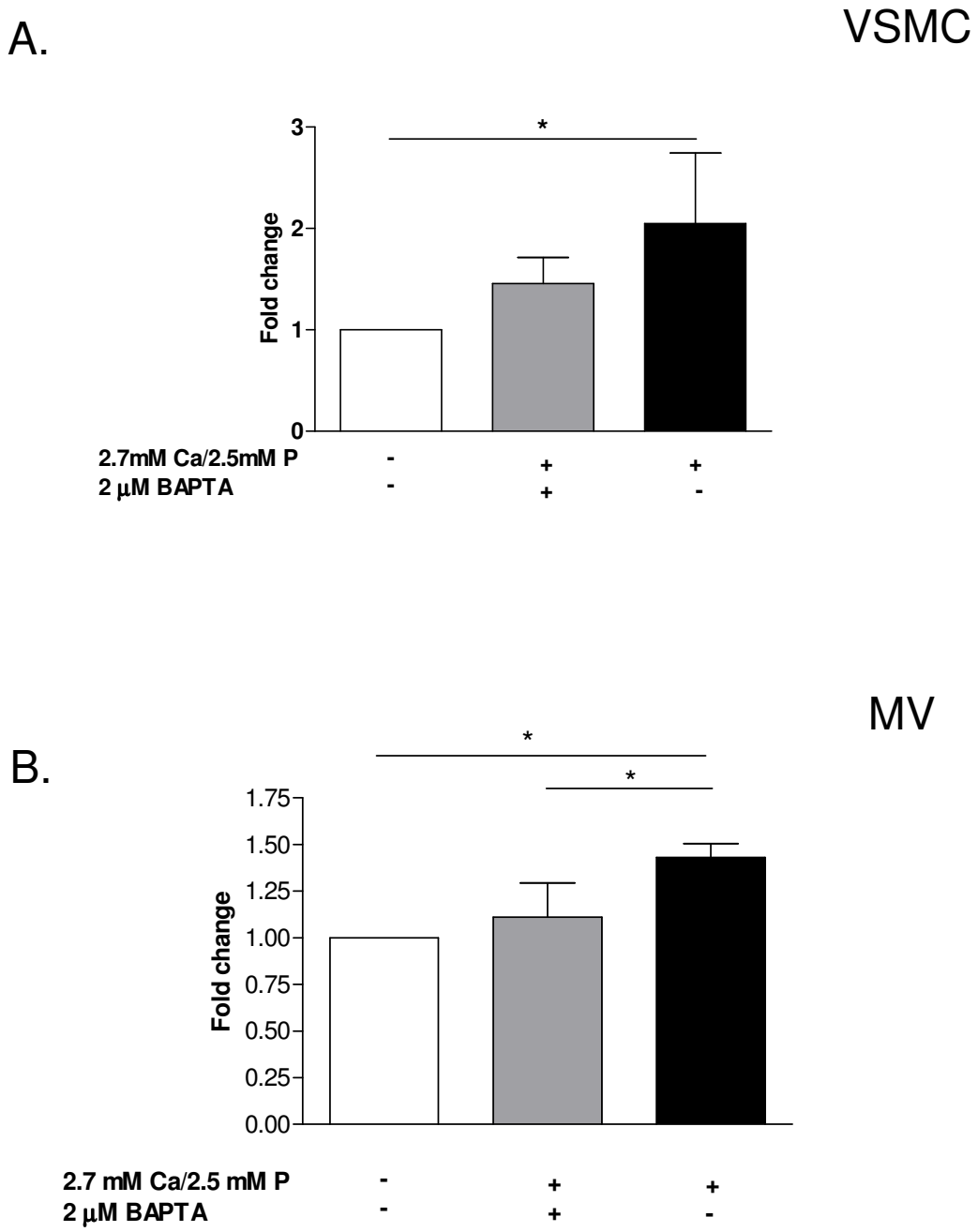

Figure VIII. Quantification of flow cytometric analyses showing changes in PS exposure on (A)the VSMC surface and (B) the surface of MV in response to calcifying media as indicated. Fold change in mean fluoresecence units (MFU) is shown. N=4, Mean +/- SD, *p<0.05.

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Supplementary Table I: Full list of proteins identified in VSMC-derived MV by 1D and LC-MS/MS.

#	Protein name	Band number	Mascot Score (total)	Peptide number	Protein molecular weight, Da	GI number
Putative Ca channels						
1	Annexin A1	9	407	13	38698	113944
2	Annexin A2	9	313	7	38588	113950
3	Annexin A5	9	592	27	35921	113960
4	Annexin A6	7	120	4	76168	113962
Putative Phosphate source						
5	5' nucleotidase, ecto	7	81	3	63898	4505467
Extracellular matrix						
6	Collagen, type VI, α1	3	226	6	109594	15011913
7	Collagen, type VI, α1	6	86	2	48497	30030
8	Collagen, type I	6	68	2	41839	30102
9	Transforming growth factor-β-induced protein, ig-h3	7	98	3	75261	4507467
10	Thrombospondin 1	6	505	16	133261	40317626
Cytoskeleton proteins						
11	Myosin, heavy polypeptide 9, non-muscle	5	2061	53	227646	12667788
12	Actin, β	8	577	35	42052	4501885
13	Cytoskeleton-associated protein 4	7	136	4	65868	74735614
	Plasma membrane proteins					
14	Aminopeptidase N	1	629	19	109842	157266300
15	Aminopeptidase N	6	545	18	109874	28678
16	Integrin, α2	1	609	13	130469	21105795
17	Integrin, α3 isoform A	3	81	3	117735	4504747
18	Integrin, αV	3	464	16	117062	4504763
19	Integrin, β1 isoform 1A	3	399	12	91664	19743813
20	Na^+/K^+ ATPase, α1	3	53	1	113936	114373
Intracellular proteins						
21	60S ribosomal protein L18	11	159	4	21735	4506607

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22	Related RAS viral (r-ras) oncogene homolog	11	75	2	23637	5454028
	Vesicle traffic					
23	Clathrin heavy chain 1	6	158	9	193260	4758012
24	Ras-related protein Rab-1A isoform 1	11	171	5	22891	4758988
25	RAB5A	11	84	2	23872	19923262
26	RAB5C	11	155	3	23781	4759020
27	RAB10	11	162	5	22755	7705849
28	RAB35	11	74	2	23296	5803135
Oxidative Stress						
29	Peroxiredoxin 1	11	94	4	22324	4505591
30	MnSOD	11	170	6	24877	34711
Protein folding/Chaperones						
31	Heat shock protein 27	10	146	4	22427	662841
Serum proteins						
32	Alpha-2-HS-glycoprotein [Bos taurus]	4	501	42	39193	27806751
33	Alpha-2-HS-glycoprotein [Bos taurus]	12	254	8	39193	27806751
34	Apolipoprotein A-I	8	283	8	30759	4557321
35	Apolipoprotein B	6	843	31	516666	4502153
36	α2 macroglobulin	6	126	6	164600	4557225
37	Fibronectin	6	62	2	260064	31397

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Supplementary Table II: Full list of proteins identified in VSMC-derived MV by 2D and LC-MS/MS.

Protein spots differentially exhibited on Cy3 compared with Silver stained gels were excised and identified using LC-MS/MS. Proteins, obtained from the gel spots which were intensively stained on the Cy3 gel when compared with the Silver gel were marked as proteins presumably located on or associated with the outer membrane surface of MV ("out"). Proteins obtained from spots which were more strong stained with silver but not visible on the Cy3 Gel were marked as proteins presumably located inside the lumen of MV ("in").

#	Protein name	2D	Percentage sequence coverage	Peptide number	Protein molecular weight, Da	GI number
Putative Ca channels						
1	Annexin A1	83	16.80%	6	38698	113944
2	Annexin A2	24 in	27.10%	11	38588	113950
3	Annexin A4	22 in	14.70%	5	35866	1703319
4	Annexin A5	65 in	27.80%	11	35921	113960
5	Voltage-dependent anion-selective channel protein 1	25 out	16.30%	4	30756	130683
Extracellular matrix						
6	Galectin-1	77 out	31.90%	4	14698	126155
Cytoskeleton proteins						
7	Myosin light polypeptide 6	68 out	32.50%	5	16912	47606436
8	Moesin	01 in	7.28%	5	67803	127234
9	WD repeat-containing protein 1	04 out	3.14%	2	66175	12643636
10	Actin, aortic smooth muscle	84	30.20%	2	42034	51316972
11	Actin, β	28 out	9.07%	3	41776	46397333
12	14-3-3 protein, ε	19 in	21.60%	6	29157	51702210
13	Calmodulin	67 in	13.40%	2	16820	49037474
14	F-actin-capping protein, subunit α1	13 in	14.30%	3	32905	1705650
15	Cofilin-1	51 in	21.70%	4	18485	116848
16	Profilin-1	61 out	26.40%	4	15036	130979
17	Coactosin-like protein	75 out	9.86%	2	15927	21759076
18	Tropomyosin α3 chain	21 out	15.10%	5	32802	54039751
19	Tropomyosin α4 chain	20 out	14.10%	3	28504	136085
20	Vimentin	29 in	4.29%	2	53634	55977767

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21	Dynactin subunit 3	43 in	10.80%	2	21102	74739637
22	Tubulin α1A chain	85	12.40%	5	50134	55977864
Intracellular proteins						
23	α-enolase	08 out	15.20%	7	47152	119339
24	L-lactate dehydrogenase A chain	23 in	15.40%	5	36671	126047
25	Nucleoside diphosphate kinase A	45 in	44.10%	2	17131	127981
26	Nucleoside diphosphate kinase B	45 in	30.90%	4	17280	127983
27	Elongation factor 1, α1	09 out	4.11%	2	50167	55584035
28	Elongation factor 1, β	18 in	10.70%	2	24746	119163
29	Eukaryotic translation initiation factor 5A-1	69 in	12.30%	2	16815	54037409
30	Histone-lysine N-methyltransferase MLL4	76 out	0.81%	2	293494	12643900
31	Stomatin-like protein 2	11 in	9.27%	2	38517	60415944
32	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β1	14 out	10.60%	2	37,360	51317302
33	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β2	14 out	10.00%	3	37314	51317304
34	Ubiquitin carboxyl-terminal hydrolase isozyme L1	35 out	25.10%	4	24806	136681
35	Proteasome subunit β type-5	72 in	8.75%	2	28463	187608890
36	Bifunctional UDP-N-acetylglucosamine 2-epimerase	87	5.12%	2	79258	45476991
37	ATP synthase subunit d, mitochondrial	40 in	18.00%	4	18474	6831494

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Oxidative Stress						
38	Peroxiredoxin 2	41 in	19.20%	4	21874	2507169
39	Peroxiredoxin 5, mitochondrial	53 in	14.00%	3	22,008	20141713
40	Glutathione S-transferase P1	36 in	10.00%	2	23339	121746
41	Protein disulfide-isomerase	63 out	19.30%	11	57100	2507460
42	Protein disulfide-isomerase A3	74 out	28.50%	15	56767	2507461
Protein folding/Chaperones						
43	T-complex protein 1 subunit ε	73 out	7.95%	5	59654	1351211
44	Peptidyl-prolyl cis-trans isomerase A	55 in	17.60%	4	17994	51702775
45	Peptidyl-prolyl cis-trans isomerase B	49 in	8.80%	2	23725	215273869
46	60 kDa heat shock protein, mitochondrial	06 out	9.08%	6	61038	129379
47	Serpin H1	09 out	21.50%	9	46424	20141241
48	Heat shock protein β1	37 in	8.29%	2	22765	19855073
49	Calreticulin	81 out	13.40%	6	48125	117501

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