Matrix vesicles (MVs) induce cell-cell communication that facilitates calcification of recipient vascular smooth muscle cells

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Abstract:

In patients with CKD and ESRD, the major risk factor for progression of arterial calcification is the presence of existing (baseline) calcification. We hypothesized that calcification of arteries is extended from calcified vascular smooth muscle cells (VSMC) to adjacent normal cells by matrix vesicle (MV) induced cell-cell communication. MV isolated from VSMC from CKD rats were co-cultured with VSMC from normal littermates and endocytosis of the vesicles by recipient cells confirmed by confocal microscopy. The addition of cellular MV with characteristics of exosomes and low fetuin-A content enhanced the calcification of recipient VSMC. Further, only cellular derived MV induced an increase in intracellular calcium ($[Ca^{2+}]_i$), NOX-1 (NADPH oxidase) and the anti-oxidant superoxide dismutase (SOD-2) in recipient normal VSMC. The increase in $[Ca^{2+}]_i$ was due to release from endoplasmic reticulum and partially attributed to the activation of both NOX and MAPK (Mitogen Activated Protein Kinase; MEK1 and Erk1/2) signaling, as inhibiting both pathways blocked the increase in $[Ca^{2+}]_i$ in recipient VSMC. In contrast, MV isolated from the media had no effect on $[Ca^{2+}]_i$, MEK1 signaling, and did not induce calcification; however media MV did increase ERK1/2 although not to the level of cellular MV, NOX-1 expression and acutely increased reactive oxygen species. Blockade of NOX activity further inhibited the cellular MV induced accelerated calcification of recipient VSMC, suggesting a potential therapeutic role of such inhibition. In conclusion, the addition of cellular derived MV from calcifying VSMC can accelerate calcification by inducing cell signaling changes and phenotypic alteration of recipient VSMC.

Introduction:

Vascular calcification is highly prevalent in chronic kidney disease (CKD) and is a major cause of morbidity and mortality(1-3). The prevalence of calcification increases with worsening kidney disease(4); by the time patients reach dialysis, 70-80 percent of patients have significant coronary artery calcification(5). On histology, medial calcification often begins as small areas within the medial layer. More advanced lesions expand to become circumferential throughout the entire medial layer(6). Risk factors for the presence of calcification in dialysis patients include older age, diabetes, and disordered mineral metabolism including hyperphosphatemia and hypercalcemia(2). However, patients with existing calcification at the start of dialysis have greater progression compared to those without calcification despite similar clinical and biochemical risk factors(7). This suggests the possibility that expansion of existing calcification occurs through different mechanisms than initiation of vascular calcification.

Studies done over the last decade have led to increased understanding of the pathophysiology of vascular calcification. The VSMC must become synthetic with increased intracellular calcium ($[Ca^{2+}]_i$)(8, 9) and downregulation of myocardin and alpha-smooth muscle actin(10), followed by de-differentiation via upregulation of the 'bone' transcription factor RUNX2(11, 12). These transformed, or de-differentiated, synthetic VSMC initiate calcification by synthesizing small 50-200 nm vesicles that initiate calcification on extracellular matrix. In bone, these vesicles are called matrix vesicles as they were identified to be an integral part of the conversion of hypertrophic chondrocytes in epiphyses of bones to develop into mineralized bone(13, 14). Over the last decade, there is also increased appreciation of the role of vesicles in cell-cell communication in non- mineralized tissues(15). Vesicles are heterogeneous and originate from the endosome or plasma membrane of cells. Although nomenclature and isolation

techniques vary, vesicles can be released through outward budding of the plasma membrane (termed shedding microvesicles) or inward budding of the endosomal membrane resulting in the formation of multivesicular bodies(16). We have previously characterized differences between vesicles isolated from the media and the cells of calcifying bovine VSMC: those from the media contain fetuin-A and do not readily mineralize, whereas those from cells do not contain fetuin-A and do mineralize(3, 17). Kapustin et al also compared vesicles from the media of calcifying human VSMC and found similar proteomic profile to that of both cellular and media vesicles from osteoblasts(18). Exosome production was increased by factors of clinical significance in CKD: increased extracellular calcium, tumor necrosis factor-α, and platelet derived growth factor BB. They further identified these vesicles to be enriched with tetraspanins (CD9, CD63, and CD81) indicating origin from multivesicular bodies, and found such multivesicular bodies in calcified human arteries(18). We and other groups have shown that the origin and content of these matrix vesicles appears to be a central determinant of their mineralization potential(3, 19). This unique function depending on content is further supported by findings that vesicles isolated from atherosclerotic plaque (macrophage derived) and medial arterial calcification also differ in content(20).

Multiple studies have demonstrated that vesicles can be taken up by recipient cells (reviewed in(21)). Given the pathologic appearance of vesicles in areas of vascular calcification in vivo and the role in calcification in vitro, we hypothesized that the transmission of vesicles from CKD cells to normal cells would facilitate calcification of the recipient cells and serve as a model of the extension or propagation of calcification observed in patients with CKD. Given the parallel pathophysiology of both physiologic and pathologic calcification, we use the term matrix vesicles (MVs).

Results:

Cellular derived, but not media derived, MVs enhanced the calcification of recipient VSMC:

We compared 4 sources of MVs: Cellular derived MVs (from CKD VSMC incubated with high phosphorus [calcifying] or with normal phosphorus [control]) or media derived MVs from calcifying or control CKD VSMC. These MVs were added to recipient normal rat VSMC as a co-culture and incubated with calcification media (high phosphorus) for 7 days. The results (Figure 1) demonstrated that both cellular sources of MVs from CKD VSMC induced calcification of the recipient VSMC. In contrast, MVs isolated from the media of cultured VSMC had no effect on calcification of recipient VSMC. Figure 2A demonstrates that the MVs isolated from both sources of cellular VSMC contained annexin II, V and VI, with higher expression in VSMC that were incubated with additional phosphorus (calcifying). In contrast, there was lower level of annexins in the MVs isolated from the media and no differences if they were from cells incubated in high phosphorus or not (2A,B). Similar to our previous finding and other reports(3, 22), vesicles isolated from the cell media contained markedly increased fetuin-A but again, little differences when the originating CKD VSMC cells were incubated with or without phosphorus (Figure 2A,B). Both cellular and media MVs contain the exosomal tetraspanins CD63, CD81 and CD9 but cellular MVs are enriched with CD63 whereas media MVs are enriched with CD81 and CD9 (Figure 2A, B). This is consistent with the report by Lotvall et al (23) that although different types of extracellular vesicles contain many common exosome-enriched markers such as tetraspanins, the relative proportions of these markers seems to vary in the different types of extracellular vesicles. Despite these differences, examination by electron microscopy (Figure 2C) showed that both cellular and media MV are around 100 mm

diameter, membrane–bound vesicles consistent with the size of exosomes as described in the literature (24). No nanotubes were identified by any imaging technique(25).

MVs are endocytosed by recipient VSMC:

To determine if VSMC can uptake MVs, we labeled MVs with the membrane fluorescent dye PKH26 and examined uptake by confocal microscopy. The results demonstrated that MVs were endocytosed by VSMC (Figure 3, red in panel A) and co-localized with Alexa 647 labeled dextran (blue panel B) but not transferrin (green panel C) by 24 h, indicating that once endocytosed, MVs become located in lysosome (Figure 3, panel E). Additional studies demonstrated MVs derived from the media are similarly endocytosed and there is no difference between MVs from VSMC incubated in normal or high phosphorus media (data not shown). Thus, the endocytosis of the MV by recipient cells is similar and not dependent on MV content.

Cellular derived MVs, but not media derived MVs, increase $[Ca]_i$ in recipient VSMC:

To determine if endocytosed MVs induced cell-signaling changes in the recipient VSMC, we examined MV mediated alterations of intracellular calcium concentration ($[Ca^{2+}]_i$) in recipient VMSC. The addition of cellular MVs (regardless of source from calcifying or control CKD VSMC) to recipient VSMC increased $[Ca^{2+}]_i$ by 60 minutes with continued increase over the 4 hrs tested (Figure 4A). In contrast, the addition of media derived MVs had no effects on $[Ca^{2+}]_i$ (Figure 4A). We therefore continued studies using only cellular derived MVs from calcifying CKD VSMC. To confirm the results, VSMC were labeled with calcium fluorescence dye Fluo-4 and MV-induced calcium transients in VSMC examined using spinning disc microscopy. The results demonstrated cellular MV increased calcium fluorescence intensity (Supplemental Figure 1) confirming our time course experiments. The MV induced increase in $[Ca^{2+}]_i$ in recipient VSMC was partially mediated by IP_3-induced $[Ca^{2+}]_i$ release as treatment

with 2-APB reduced MV-induced increase in $[Ca^{2+}]_i$ (indicating release of calcium from sarcoplasmic reticulum) but blocking external entry of calcium with L-type calcium channel with verapamil had no effect (Figure 4B).

MVs activate MAPK signaling in recipient VSMC:

To determine the role of MVs on MAP kinase signaling in VSMC, cellular or media MVs were isolated from calcifying CKD VSMC. First cellular MVs were incubated with normal recipient VSMC at various time points and the activation of MAP kinase assessed using PathScan MAP kinase multi-Target Sandwich ELISA kit. The activity of phospho-Erk1/2 and phospho-MEK1 was increased at 30 min and remained similarly increased at 2h and 4h in VSMC (30 minute time point shown in Figure 5A). However, MVs had no significant effect on activation of phosphor-p38 MAPK and phosphor-APPK/JNK. To compare the role of cellular MV and media MV on the activation of MAPK in recipient VSMC, Western blot was used and results confirmed cellular MV induced phosphorylation of Erk1/2 (Figure 5B) and MEK1 (Figure 5C). In contrast, media MV had no effect on phosphorylation of MEK1 and slightly increased phosphorylation of Erk1/2. Furthermore, inhibition of MAP kinase activity by pre-incubating the normal VSMC with MEK1 and Erk1/2 inhibitor U0126 decreased cellular MV-induced elevation of $[Ca^{2+}]_i$ (Figure 5D). The effect of inhibition of MAPK on calcification could not be assessed due to toxicity to cells with prolonged incubation.

MVs modulate the expression of genes involved in VSMC differentiation and calcification in recipient VSMC:

The co-culture of cellular CKD MVs with VSMC decreased the recipient VSMC gene expression of smooth muscle actin-22 (SM22a) and increased expression of angiotensin receptor 1 (AT1R) at day 7 (Figure 6A) but not at day 1 or 3. The addition of MVs had no effect on the

recipient VSMC expression of myocardin at any time point (Figure 6A). MVs also increased the expression of bone morphogenic-2 (BMP-2) in recipient VSMC at day 7, but not day 1 and 3, and had no effect on the expression of RUNX2 or osteocalcin (Figure 6B). These results demonstrate that the addition of MV alter some, but not all, of the genes known to be important in calcification after 7 days but not at earlier time points.

MVs uptake induces NOX signaling in recipient VSMC:

Altered intracellular calcium signaling can induce changes in mitochondrial function and oxidative stress and vice versa. We first examined the expression of NOX isoforms in cultured VSMC and found that the expression of NADPH oxidase (NOX)-1 and NOX-4 were increased during calcification of CKD VSMC (Supplemental Figure 2). We thus examined the effect of cellular MV on the expression of NOX-1 and NOX-4 in recipient VSMC after 1, 3 and 7 days. The expression of NOX-1 in recipient normal VSMC was increased at all three time points (Figure 7A, top panel), but there was no effect on NOX-4 at any time point (not shown), the latter known to be constitutively active in VSMC(26). However, MV did not increase NOX1 protein levels at any time point (data not shown). We then examined the expression of the anti-oxidant superoxide dismutase, and found the addition of cellular MV to VSMC increased the expression of SOD-2 at day 3 and 7, but not at day 1 (Figure 7A, bottom panel). However, there was no increase in expression of SOD-1 in VSMC at any of the three time points (data not shown). Assessment of mitochondrial function in cellular MV-VSMC co-culture by western blot using total OXPHOS cocktail antibodies revealed no changes in any of the mitochondrial subunits (Supplemental Figure 3). We then examined the expression of NOX1 in VSMC co-cultured with media derived MV and found increased NOX-1 but no change in SOD2 after 3

days [For NOX-1 expression, No MV=1.35±0.10; media MV=2.23±0.39 ($p<0.01$); For SOD2 expression, No MV=0.85±0.07; media MV=1.07±0.22 (NS)].

To determine the role of NOX activity in cellular MV-mediated signaling and calcification in recipient VSMC, cellular MVs were added to normal VSMC in the presence or absence of the specific NOX1/4 activity inhibitor, GKT137831 and $[Ca^{2+}]_i$ and MAPK signaling determined. Inhibition of NOX activity reduced cellular MV induced increase in $[Ca^{2+}]_{i.}$ (Figure 7B) but had no effect on MAPK signaling (For phosphor-MEK1: MV=1.37±0.02 AU; MV+GKT137821=1.40±0.03 AU). Confirming the importance of NOX activity in calcification, the addition of GKT137831 to co-cultures partially reduced cellular MV induced calcification of recipient normal VSMC (Figure 7C).

Discussion:

MVs have a critical role in the initiation of mineral deposition in skeletal tissues. In the current study we demonstrated endocytosis of cellular derived MV isolated from CKD VSMC by recipient normal VSMC with a rise in $[Ca^{2+}]_i$, increase in MEK1 and ERK1/2 MAPK signaling, and accelerated calcification. In contrast, MV isolated from the media had no effect on $[Ca^{2+}]_i$, MEK1 signaling, and did not induce calcification; however media MV did increase ERK1/2 although not to the level of cellular MV. We further demonstrated that inhibition of MEK1/ ERK1/2 signaling with the specific inhibitor U0126 reduced cellular MVs-induced alteration of [Ca2+]i in recipient VSMC. In contrast, we did not see a change in p38 and JNK signaling. Taken together, these results suggest that MEK1 is the predominant MAPK signaling pathway for both the increased $[Ca^{2+}]_i$ and calcification in the cellular MV-VSMC co-cultures(27).

Previous studies have demonstrated the MAPK-ERK signaling is an important pathway in VSMC proliferation/differentiation(28) and in de-differentiation of smooth muscle cells to osteochondrogenic (RUNX2 expressing) cells in arteries(29), whereas phosphorus induced calcification acts primarily through p38(30, 31). In the present study, we found some changes in gene expression consistent with a switch from a vascular to an osteoblast like phenotype after the addition of MV to recipient normal VSMC (downregulation of sm22α, and upregulation of BMP-2 and AT1R). However, these changes were only observed at 7 days, and thus unlikely to be due to the immediate MEK1/ERK signaling observed within 30 minutes of the addition of MV, or be the major mechanism by which calcification is enhanced by cellular MV. However, it is likely that the late differentiation of the VSMC is still critically important in calcification and perhaps the addition of MV augments these changes through additional mechanisms.

Alteration of $[Ca^{2+}]_i$ induced endoplasmic reticulum (ER) stress may also be important in the pathogenesis of vascular calcification. The ER stress markers Grp78, Grp94, and CHOP were found in calcified artery from rats treated with vitamin D which is known to increase in $[Ca^{2+}]_i$ and calcification in VSMC(32). Such mitochondrial stress leads to excess H_2O_2 and O_2- that can modify sulfhydryl groups of cysteine residues of signaling pathways and calcium transport proteins(33) including the sarcoplasmic reticulum calcium ATPase (SERCA)(34, 35), phospholipase C (PLC)- inositol1,4,5 trisphosphate (IP_3)(36) and the ryanodine receptor (RyR)(37). Our studies confirmed that the rise in intracellular calcium was from an endoplasmic source as 2-APB, an inhibitor of IP_3, blocked the increase in $[Ca^{2+}]_i$ in response to uptake of cellular MV. We have previously demonstrated that freshly isolated VSMC from the CKD animals used in this study have a progressive rise in $[Ca^{+2}]_i$ with increasing severity of CKD(9). Such changes may be induced by many uremic toxins, including PTH(38), FGF23(39, 40), and

angiotensin II(41), all of which are elevated in patients with CKD and known to alter VSMC phenotype and/or calcification.

In contrast to the late changes in cell differentiation marker RNA expression in recipient VSMC, changes in NOX1 expression were observed by day 1 in the cellular MV-VSMC co-cultures and changes in $[Ca2+]_i$ can upregulate NOX1 and vice versa(42). Given the absence of changes in protein expression of NOX-1, we inhibited NOX activity with GKT137831 and reduced calcification. This suggests an important role of NOX in cellular-MV induced calcification further supported by our data demonstrating GKT137831 also blocked the increase in $[Ca2+]_i$ (but not MAPK) in the cellular MV- VSMC co-cultures. The family of NADPH oxidases generates superoxide and other ROS species at both the plasma and endoplasmic reticulum membranes with variable expression of isoforms depending on the cultured condition and species(27). The generation of ROS, in turn, alters $[Ca^{2+}]_i$, and mitochondrial function(42, 43) but we did not see a change in downstream mitrochondiral components with the uptake of cellular MV by VSMC. This may be due to counter effects of anti-oxidants as we observed the mitrochondrial SOD-2 was upregulated by 3 days in cellular VSMC MV co-cultures. We similarly saw an upregulation of NOX-1 RNA expression in media MV-VSMC co-cultures, although media MV did not induce a rise in $[Ca^{2+}]_i$, MEK, SOD-2 expression or calcification. Thus, despite similar effects on NOX-1 RNA expression by both the cellular and media MV, the downstream signaling and calcification differ. These results suggest distinct intracellular trafficking of the media MV from cellular MV prior to ending up in lysosomes, and that these differences appear important in calcification. Additionally, the differences in results may also be due to differences in NOX-1 activity, as changes in expression do not always equate with activity. More work is needed to fully clarify the downstream pathways.

We have previously characterized matrix vesicles isolated from cell lysate and cell culture media of bovine VSMC(3), demonstrating that MVs isolated from the cell culture media contain high concentrations of fetuin-A, whereas vesicles isolated from cells do not. These findings were confirmed in the present study in rat VSMC and the presence of fetuin-A may be another explanation for why media vesicles do not induce calcification. We also found that the fetuin-A containing rat vesicles lacked the tetraspanin exosome marker CD63, an important exosome marker used to isolate exosomes from human fluids(44). Thus, the media vesicles in cell culture may not equate with circulating vesicles isolated using this technique from human fluids. In contrast, our cellular derived matrix vesicles were characteristic of exosomes derived from multivesicular bodies. Recently Kapustin et al identified such bodies in areas of calcification of arterial rings incubated with high calcium or from that of dialysis patients(18). Fetuin-A is a known inhibitor of VSMC and osteoblast mineralization in vitro(45-47) and, when present in vesicles renders them incapable of calcium uptake(46). Fetuin-A prevents the initial calcium apatite formation by preferentially trafficking the calcium and phosphorus into fetuin-A-containing calciprotein particles in the circulation(48). The differences in $[Ca^{+2}]_i$ in response to MVs that contain and do not contain fetuin-A in the present study may be one mechanism by which differences in calcification potential of these MV occur. We have previously demonstrated that the calcification activity of MVs can be inhibited by decreasing $[Ca^{2+}]_i$ with the annexin calcium channel inhibitor K201, either in the VSMC from which the MVs are isolated, or the MVs themselves(3, 17).

In summary, we have demonstrated that cellular derived MVs isolated from CKD rat VSMC can facilitate the calcification of recipient VSMC from normal rats. Both cellular and media MVs can be endocytosed by recipient normal VSMC. However, only cellular MV

induced an increase in $[Ca^{2+}]_i$ from endoplasmic reticulum in the recipient VSMC. The increase in $[Ca^{2+}]_i$ is partially attributed to the activation of both NOX and MAPK signaling, as inhibiting either pathway blocked the increase in $[Ca^{2+}]_i$ in recipient VSMC . Blockade of NOX activity further inhibited the cellular MV induced accelerated calcification of recipient VSMC, but did not completely abrogate calcification suggesting other pathways are important. In contrast, media MV did not induce an increase in $[Ca^{2+}]_i$ in recipient VSMC perhaps because the increase in NOX was not offset by an increase in SOD-2. Understanding the differences in the cell signaling induced by different forms of MV that lead to the presence or absence of calcification in neighboring cells will be important to stop the progression of calcification in vivo.

Methods:

Animal models and cell culture:

Primary rat vascular smooth muscle cells (VSMC) were isolated from a model of Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD), the Cy/+ rat or its normal littermates. This model spontaneously develops all three manifestations of CKD-MBD: biochemical abnormalities, extraskeletal calcification, and abnormal bone(49, 50). VSMC were isolated from the descending thoracic aorta of CKD or normal rats by the explant method as previously described(51). To induce calcification, VSMC were treated with calcification media (5 mM β-glycerophosphate [which is converted to phosphorus], 1 U/ml fetal alkaline phosphatase and 15% FBS)(51) compared to control cultures without the β-glycerophosphate but with normal media phosphorus levels. Co-culture experiments (see below) were always in the presence of β-glycerophosphate.

Matrix vesicle (MV) isolation:

Cellular derived MVs were isolated from CKD rat VSMC by collagenase digestion with sequential centrifugation as previously described(3). In brief, cells were incubated with crude collagenase (500 U/ml, type IA, Sigma) in a solution of 0.25 M sucrose, 0.12 M NaCl, 0.01 M KCl and 0.02 M Tris buffer, pH 7.45, at 37^{o}C for 3 hrs. The digests were centrifuged at 800 g and 30,000 g to remove cell debris, apoptotic bodies and microsomes, respectively. The supernatant was centrifuged at 250,000 g to pellet the MVs followed by resuspension in TBS (pH 7.6) with 0.25 M sucrose. In some experiments, MVs were also isolated from the cultured cell media (Media MVs) as we have previously published(3). The media was decanted and spun at 30,000 g for 10 min followed by centrifugation at 250,000 *g* for 30 min at 4°C and MV isolated from the supernatant. The MVs were quantified by protein concentration (Bio-Rad).

Western blotting:

Western blotting was performed as previously described(3). The blots were incubated with antibody against annexin II, V and VI, CD63, CD81 and CD9 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or fetuin-A (1:2000, a gift from Dr. Willi Jahnen-Dechent, University Hospital, Aachen, Germany) overnight at 4^{o}C followed by incubating with peroxidase conjugated secondary antibody (1:5000 dilution), and immunodetection with the Enhanced Chemiluminescence Kit (Amersham, Piscataway, NJ). The band intensity was analyzed by ChemiDoc MP Imaging System (Imaging Lab 4.0, Bio-Rad, Richmond, CA) and normalized to total protein expression using Ponceau S.

Transmission electron microscope (TEM) for matrix vesicles:

TEM was performed by the EM center at Indiana University School of Medicine. Briefly, 20 ug of cellular or media MV were fixed with 10% glutaraldehyde and then 300 mesh nickel formvar/carbon coated grids (Electron Microscopy Sciences, Hatfield, PA) were placed

under the matrix vesicle solutions and allowed to absorb over a weekend at 4°C. The grids were then taken out of the solution and allowed to dry for approximately 1 minute, then negative stained for 10 seconds using Nanovan (Nanoprobes, Inc, Haphank,NY). The grids were viewed on a Tecnai Spirit (Thermofisher Scientific, Hillsboro, OR) and images taken with a CCD camera (Advanced Microscopy Techniques, Danvers, MA).

MV-VSMC co-culture:

To determine if MVs from CKD animal derived VSMC enhanced calcification of recipient normal animals derived VSMC, co-culture experiments were done. The normal VSMC were incubated with or without 10 μg of MVs, always in the presence of β-glycerophosphate (to provide phosphorus which is needed for calcification) for up to 7 days. The media was removed and cells incubated in 0.6N HCl for 24 hours and calcification determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific) as previously described(3). In some experiments, MV-VSMC co-culture were treated with or without inhibitor of 1,4,5-trisphosphate (IP3)-receptor (2-APB, Calbiochem, Darmstadt, Germany), MAPK inhibitor U0126 (Cell Signaling Technology, Danvers, MA) or NOX1/4 inhibitor GKT137831 (BioVisions, Inc, Milpitas, CA).

MV endocytosis in recipient VSMC:

MVs were labeled with membrane fluorescent dye PKH26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma, St Louis, MO) and added to cultured recipient VSMC, and the uptake examined by confocal microscopy at various time points by MRC-1024 laser-scanning confocal microscope (Bio-Rad) as previously described(52, 53). To determine the co-localization of MVs with endosomes or lysosomes, Alexa 488 labeled transferrin (labels recycling endosomes) and

Alexa 647 labeled dextran (labels lysosomes) were also added to the co-cultures (Molecular Probe).

MV mediated alteration of intracellular calcium ($[Ca]_i$):

To determine if MVs alter the $[Ca]_i$ in recipient VSMC, normal VSMC were seeded in 96-well culture plates and labeled with the calcium Rhod-3 Calcium Imaging kit (Molecular Probe, Carisbad, CA) for 30 minutes. MVs were then added to the VSMC and the acute change in $[Ca^{2+}]_i$ in VSMC were assessed by fluorescence at various time points up to 4 hrs using CLARIOstar high performance microplate reader (BMG LABTECH Inc, Cary, NC). Results were confirmed with spinning disc microscopy(54).

Effect of MVs on MAP kinase signaling in VSMC:

To determine if MVs affect MAP kinase signaling, recipient VSMC were incubated with or without 10 µg MVs for 30 min, 2h and 4 h at 37^{o}C and total protein from the co-culture isolated using lysis buffer as previously described(51). The activation of MAP kinase was assessed using PathScan MAP kinase multi-Target Sandwich ELISA kit (Cell Signaling Technology, Danvers, MA). To confirm the MAP kinase signaling by cellular MV and media MV, Western blot analyses were performed. Briefly, 20 µg of protein was loaded on 10% SDS-PAGE and the blots were incubated with antibody against Phospho-MEK1 or Phospho-Erk1/2 (1:500, Cell Signaling Technology, Danvers, MA) overnight at 4^{o}C followed by incubating with peroxidase conjugated secondary antibody (1:5000 dilution), and immunodetection with the Enhanced Chemiluminescence Prime Western Blot Detection Reagent (Amersham, Piscataway, NJ). For loading control, western blot was also performed using antibodies against total MEK1 or total Erk1/2 (1:100, Cell Signaling Technology, Danvers, MA). The band intensity was analyzed by ChemiDoc MP Imaging System (Imaging Lab 4.0, Bio-Rad, Richmond, CA) and

MPA kinase activation was quantified by normalizing phosphorylated MAP kinase to total MAP kinase.

RNA isolation, quantification and real-time PCR:

Total RNA from MV-VSMC co-culture was isolated using miRNeasy Mini Kit (Qiagen). Target-specific PCR primers were obtained from Applied Biosystems. The gene expression of bone morphogenic protein 2 (BMP-2), RUNX-2, osteocalcin, Sm22α, myocardin, NADPH oxidase isoform 1 and 4 (NOX1 and 4), angiotensin II type I receptor (AT1R) and superoxide dismutase (SOD) 1 and 2 was analyzed by real time PCR using Taqman gene expression assay system (TaqMan MGP probes, FAM dye-labeled , Applied Biosystems, Foster City, CA) using ViiA 7 systems(10). The cycle number at which the amplification plot crosses the threshold was calculated (C_T), and the $\Delta\Delta C_T$ method was used to analyze the relative changes in mRNA expression and normalized by beta-actin as previously described(10).

Statistics:

Statistical analysis was conducted by ANOVA and within group comparisons by Fisher's post hoc analysis. The results are expressed as means ± SD, with $p<0.05$ considered significant (StatView, SAS Institute, Cary, NC).

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Figure Legends:

Figure 1: MV induced calcification of recipient VSMC:

MVs were isolated from four sources of MVs: Cellular derived MVs (from CKD VSMC incubated with high phosphorus (5 mM β-glycerophosphate) = calcifying or Cal = black bars] or with normal phosphorus (no β-glycerophosphate) [control = Ct = white bars]) or media derived MVs from calcifying or control CKD VSMC. Normal VSMC were incubated alone (hatched bar), or with cellular or media derived MV in the presence of β-glycerophosphate. Only the cellular origin of MV induced calcification of the recipient normal VSMC. Data are shown as mean ± SD (n = 3 MV sets from 3 CKD rats, with three cell cultures from each MV set for final n of 9). * $p < 0.05$, Cellular MV vs. VSMC alone or VSMC + media derived MV.

Figure 2: Comparison of content of MV isolated from cells or media:

MVs isolated from the same four sources as in Figure 1 were analyzed for content of annexin II(36 kDa), annexin V (36 kDa), annexin VI (47-51 kDa) , fetuin-A (59 kDa), CD63 (core protein, MV 26 kDa) , CD81 (22-26 kDA) and CD9 (24 kDa) by Western blot (A) with quantification of band intensity normalized by Ponceau S (B). MVs isolated from cells had increased expression of annexins and CD63, but neglible fetuin-A compared to that from MVs isolated from media. Isolation from cells in high phosphorus (calcifying) media in general increased expression. In contrast, in the MVs isolated from the media, there was high fetuin-A content, high levels of CD81 and CD9 and no differences when isolated from VSMC with and without calcifying (high phosphorus) media. TEM showed that both cellular (Figure 2C, left panel) and media MV (Figure 2C, right panel) show uniform size of 100 mm diameter, membrane–bound vesicles. Ct = MV isolated from CKD VSMC in normal phosphorus media; Cal = MV isolated from CKD VSMC in high phosphorus media. Data are shown as mean ± SD (n = 3 separate experiments). * $p < 0.05$, Ct MV vs. Cal MV same source (Cellular MV or media MV); # $p<0.05$, Cellular MV vs. media MV, same condition (control or calcifying/high phosphorus). 200 nm (C).

Figure 3: Both cellular and media MV are endocytosed by recipient VSMC:

MV isolated from CKD VSMC incubated with high phosphorus media and then labeled with the membrane dye PKH26. The MVs were co-cultured with VSMC (A), Alexa 647 labeled dextran (B) and Alexa 488 labeled transferrin (C), and imaged by confocal microscopy (60X objective, scale bar=50 μm). After 24 hours, endocytosis was observed with co-localization of the MV dye with dextran (purple, E), indicating MV colocalize with lysosomes. We also examined cellular MV isolated from VSMC with normal phosphorus (Ct) and media derived MV and found similar patterns of endocytosis.

Figure 4: MVs differ in their ability to increase intracellular calcium $[Ca^{2+}]_i$ in recipient VSMC:

A: MV from cellular or media derived CKD VSMC in normal (Ct) or high phosphorus media (Cal) were added to VSMC labeled with the calcium Rhod-3 and $[Ca^{2+}]_i$ assessed by fluorescence at various time points up to 4 hrs using CLARIOstar high performance microplate reader. The results demonstrate that cellular derived MV, regardless of phosphorus content, induce a progressive rise in $[Ca^{2+}]$i whereas media derived MV do not. B: Cellular derived MV from calcifying CKD VSMC were co-cultured with VSMC with and without the IP3 inhibitor 2APB (10 μM) or the L-type calcium channel inhibitor verapamil (10 μM) for 4 hrs and $[Ca^{2+}]_i$ assessed. The results show attenuation of MV-mediated induction of $[Ca^{2+}]$i with 2APB but not verapamil, indicating the rise in calcium was due to release from intracellular stores. Data are shown as mean ± SD (n = 3 MV sets from 3 CKD rats, with three cell cultures from each MV set for final n of 9). (A): * $p < 0.05$, Cellular MV vs. media MV or no MV, Ct MV or Cal MV; (B): * $p < 0.05$, MV vs. no MV, treatment or no treatment; # $p<0.05$, MV vs. MV +2-APB.

Figure 5: MVs activate MAPK signaling in recipient VSMC:

MV isolated from cellular derived CKD VSMC in high phosphorus media were added to normal VSMC for 30 minutes and total protein from the co-culture isolated. The activation of MAP kinase was first assessed using PathScan MAP kinase multi-Target Sandwich ELISA kit. The results demonstrated there was an increase in phospho-MEK1 and phosphor-Erk1/2 compared to VSMC without MV (Figure 5A). Western blot confirmed cellular but not media MV-induced phosphorylation of ERk1/2 (Figure 5B) and MEK-1 (Figure 5C) (normalized by total Erk1/2 and MEK) in recipient VSMC. Pre-incubation of the recipient VSMC with the MAPK (MEK/ERK) inhibitor U0126 (10 μM) for 12 hours partially attenuated MV-induced a rise in $[Ca^{2+}]i$ in recipient VSMC (Figure 5D). Data are shown as mean ± SD (n = 3 separate experiments). *$p < 0.05$, VSMC vs. MV+VSMC, cellular or media MV; #$p<0.05$, cellular MV vs. media MV;. For Figure 5D; *$p < 0.05$, no MV vs. MV; #$p<0.05$, MV vs. MV+U0126.

Figure 6: MV induces late changes in genes involved in differentiation and calcification in recipient VSMC:

The addition of MV derived from CKD VSMC to recipient normal VSMC induced downregulation of the vascular smooth muscle marker sm22α (A), and upregulation of AT1R (A) and BMP-2 (B) at 7 days. No changes were observed at days 1 and 3 for these genes, and other genes were unaffected. Thus, MV induced late changes consistent with an osteoblast phenotype. Data are shown as mean ± SD (n = 3 MV sets from 3 CKD rats, with three cell cultures from each MV set for final n of 9). * $p < 0.05$, MV vs. no MV.

Figure 7: MVs altered oxidative stress in in recipient VSMC:

The addition of cellular MV from calcifying CKD VSMC to normal VSMC induced changes in NOX-1 expression by 24 hrs that continued over time (top panel) and an increase in the anti-oxidant SOD-2 beginning at day 3 and continuing over time (bottom panel). Data are shown as mean ± SD (n = 3 MV sets from 3 CKD rats, with three cell cultures from each MV set for final n of 9). * $p < 0.05$, MV vs. no MV.

Figure 8: The role of NOX activity in cellular MV mediated cell signaling and calcification in recipient VSMC:

To determine the role of NOX activity in cellular MV-induced cell signaling and calcification, cellular MV were added to recipient VSMC in the presence or absence of NOX1/4 activity blocker GKT137831 (4 μM) and $[Ca]_i$ and calcification determined. The results demonstrated that blockade of NOX1/4 activity attenuated the cellular MV induced rise in $[Ca]_i$ (A) and calcification of recipient VSMC (B). Data are shown as mean ± SD (n = 3 MV sets from 3 CKD rats, with three cell cultures from each MV set for final n of 9). * $p < 0.05$, cellular MV vs. no MV. # $p<0.05$, MV vs. MV+137831.