ROLE OF INORGANIC PHOSPHATE IN VESICLE RELEASE FROM OSTEOGENIC CELLS

by

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Extracellular vesicles (EV) are membrane-enclosed microstructures released from the cell. EV can transport cargo—such as DNA, RNA, and proteins—into the extracellular space and act as a form of intercellular communication. Matrix vesicles (MV) are a unique type of EV produced by mineralizing cells. In previous studies, utilizing *in vitro* models of pre-odontoblasts and vascular smooth muscle cells, it was determined that phosphate induces the release of MV and alters MV composition. We hypothesized that elevated phosphate alters the composition of MV through the alteration of gene expression. To determine whether this hypothesis holds true or not, 17IIA11 cells, a pre-odontoblast cell line, were exposed to elevated phosphate for 24h, 48h, and 72h. RNA and MV were then collected for further analysis. Using nanoparticle tracking analysis, we determined that the size distribution of the MV was not altered by the phosphate. Next, to evaluate a possible mechanism driving MV composition change, we compared the effect of phosphate on gene expression to the effect of phosphate on MV composition. No correlation was identified between changes in gene expression and changes in MV composition. These findings suggest that phosphate does not alter the composition of MV through altered gene expression. Future studies should focus on other mechanisms that may drive MV composition changes.
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1.0 INTRODUCTION

1.1 EXTRACELLULAR VESICLES

Extracellular vesicles (EV) are membrane-enclosed microstructures released from the cell. EV can transport cargo—such as DNA, RNA, and proteins—into the extracellular space and act as a form of intercellular communication. All cells release EV and, the cargo of EV often retains its biological function. There are several types of EV, characterized based on their biogenesis or release pathways; the most well-defined types of EV are apoptotic bodies, exosomes, microvesicles (Figure 1) (György, Szabó et al. 2011, Yuana, Sturk et al. 2013, Colombo, Raposo et al. 2014, Yanez-Mo, Siljander et al. 2015, Zaborowski, Balaj et al. 2015, Hasegawa 2018).

Apoptotic bodies are released after the initiation of apoptotic cell death. This type of EV involves blebs directly from the plasma membrane with a diameter ranging in size from 500 nanometers (nm) to 1 micrometer (µm). Exosomes, another commonly studied EV subtype, are formed through the fusion of multivesicular bodies containing smaller vesicles and the plasma membrane, which results in the release of the smaller vesicles (exosomes) ranging in diameter from 40nm to 120nm. A third EV subtype, microvesicles, bud directly from the plasma membrane, contain cytoplasmic cargo, and range in diameter from 50nm to 1 µm (Roberts, Stewart et al. 2004, Thery, Amigorena et al. 2006, Julien, Khoshniat et al. 2009, György, Szabó

Current research in the field suggests that the function of EV is related to the composition of the cargo. An early study by Théry et al. (2001) evaluating the composition of EV released from dendritic cells established the first extensive protein map of an EV population, showing that the composition of vesicles released by dendritic cells could impart an immunostimulating function (Théry, Boussac et al. 2001). This study suggests that EV act as a form of cell-cell communication.

Other studies of EV have theorized that the roles of EV are far more varied than previously thought. For example, EVs have been found to contain and carry the receptors of many growth factors, such as TNF-α, EGF, and FGF. The extracellular matrix (ECM) retains these growth factors, and they can be released during ECM remodeling. The release of these molecules can regulate cell proliferation, migration, and morphogenesis. EV containing the receptors for these factors suggest that they may play a role in regulating their release. EVs have also been found to contain matrix metalloproteinases with proteolytic activity and EV may play a role in alteration of EV content and ECM degradation (Rackov, Garcia-Romero et al. 2018). These findings indicate the diverse roles EV can play in cellular function.
1.2 MATRIX VESICLES

In bone biology, we find a fourth well-defined type of EV: matrix vesicles (MV). These range in size from approximately 80nm to 220nm and bud from specific regions of the plasma membrane. MV are a unique set of EV that anchor to the ECM and initiate the mineralization of the ECM through the nucleation of hydroxyapatite. They are known to be released from mineralizing cells such as hypertrophic chondrocytes and osteoblasts. Furthermore, EV derived from arterial endothelial cells, vascular smooth muscle cells, and macrophages have been associated with the pathological mineralization of the arteries, a dangerous complication of cardiovascular diseases. (Anderson 1967, Boyan, Schwartz et al. 1989, Wuthier and Lipscomb 2011, Shapiro, Landis et al. 2015, Chaudhary, Kuzynski et al. 2016, Bottini, Mebarek et al. 2018, Chaudhary, Khalid et al. 2018, Hasegawa 2018).

The composition of MV released from stem cells undergoing osteogenic differentiation (using an osteogenic medium composed of an inorganic phosphate (P_i) source and ascorbic acid)
has been previously studied. These MV were shown to be enriched in the proteins involved in mineralization such as tissue non-specific alkaline phosphatase (TNAP), calcium phosphate transporters, and annexesins (calcium transporters). These vesicles are also enriched in ESCRT (endosomal sorting complexes required for transport) proteins, suggesting they are packaged and sorted through the endosomal pathway before being released into the ECM (Thouverey, Malinowska et al. 2011).

Studies of cartilage and arteries have also identified MV as being associated with pathological mineralization. However, one study comparing the proteomes of MV released from normal and osteoarthritic cartilage found that only a small number of proteins, mostly involved in inflammation, were different between the two groups (Rosenthal, Gohr et al. 2011). Another study evaluating MV from normal cartilage samples identified full-length mRNA contained in MV for collagen II, aggrecan, and ANKH, an inorganic pyrophosphate transport regulator. When chondrocytes were then exposed to these MV, the MV were internalized, and there was an observed change in gene expression by these genes (Mitton, Gohr et al. 2009).

While the composition of MV released from osteoblasts, chondrocytes, and cartilage has been evaluated, our group was the first to evaluate the composition of MV released from odontoblasts, which undergo physiologic mineralization, as well as vascular smooth muscle cells, which undergo pathologic mineralization. From these studies, we identified $P_i$ as an initiator of MV release from these cells (Figure 2) (Anderson, Garimella et al. 2005, Yuana, Sturk et al. 2013, Chaudhary, Kuzynski et al. 2016, Chaudhary, Khalid et al. 2018).
1.3 PHOSPHATE AND MINERALIZATION

Pi is one of the most important and abundant ions present in the body. It is part of the DNA backbone, stores energy in the form of ATP, and activates enzymes through phosphorylation. It is a major component of hydroxyapatite and, most importantly for our study, acts as a signaling molecule, particularly in the initiation of mineralization (2006, Khoshniat, Bourgine et al. 2011, Chaudhary, Kuzynski et al. 2016, Chaudhary, Khalid et al. 2018).

Elevated Pi is provided, in part, to the cell locally through conversion of pyrophosphate to Pi by TNAP, and it signals for osteogenic differentiation of stem cells (Ciancaglini, Yadav et al. 2010, Wuthier and Lipscomb 2011, Millan 2013, Huesa, Houston et al. 2015, Fakhry, Roszkowska et al. 2017). Unpublished data from our lab has indicated that Runt-related transcription factor 2 (Runx2), the master regulator of osteogenic differentiation, is up-regulated by elevated Pi in a pre-odontoblast cell line (data not shown), suggesting that Pi may regulate osteogenic differentiation through alteration of gene expression. Furthermore, a study utilizing MC3T3-E1 cells (a pre-osteoblastic cell line) showed significant modulation of protein expression, specifically in functions related to cell cycle regulation, proliferation, and DNA synthesis, when exposed to elevated Pi (Conrads, Yi et al. 2005).

Several studies have also identified alteration of gene expression of proteins related to mineralization when osteogenic progenitor cells and osteosarcoma cells were exposed to elevated Pi. These proteins include osteopontin (OPN), and dentin matrix protein 1 (DMP1) (Beck, Zerler et al. 2000, Beck, Moran et al. 2003, R. 2003, Conrads, Yi et al. 2005, Khoshniat, Bourgine et al. 2011). In an unpublished RNA-seq analysis of 17IIA11 cells (17A, a pre-odontoblast cell line) exposed to elevated Pi, we identified significant alteration of gene expression of genes known to be involved in the mineralization of the ECM (data not shown).
This finding provides further evidence supporting Pi’s role as a signaling molecule involved in mineralization.

However, very little is understood about the pathways through which Pi acts. The only known mediators of phosphate signaling include Extracellular signal-regulated kinase 1 and 2 (ERK1/2). Members of the Pi transporter (PiT) family are the only known transporters of Pi into the cell. Of further interest to this study is the fact that many of the proteins known to regulate local concentrations of phosphate (TNAP, PiT-1) are known to be enriched in MV (Beck, Zerler et al. 2000, Beck, Moran et al. 2003, R. 2003, Conrads, Yi et al. 2005, Julien, Khoshniat et al. 2009, Khoshniat, Bourgine et al. 2011, Wuthier and Lipscomb 2011, Bottini, Mebarek et al. 2018). Another intriguing observation is that the protein products of genes affected by elevated Pi are known to reside in the ECM during mineralization and are important regulators of mineralization (DMP1 and OPN) (McKee and Nanci 1996, Beck, Zerler et al. 2000, R. 2003, Khoshniat, Bourgine et al. 2011, Chaudhary, Khalid et al. 2018). As Pi acts as a signaling molecule, and many regulators of Pi and mineralization are enriched in MV, we decided to investigate whether Pi played a role in MV composition and release from osteogenic cells and found that it did (Figure 2).

1.4 PHOSPHATE AND EXTRACELLULAR VESICLE RELEASE FROM OSTEOGENIC CELLS

MV have long been thought to be the nucleation sites of hydroxyapatite crystals (Wuthier and Lipscomb 2011, Alves, Eijken et al. 2014, Chaudhary, Kuzynski et al. 2016, Bottini, Mebarek et al. 2018, Chaudhary, Khalid et al. 2018, Hasegawa 2018). In previous work, we evaluated the
role of P_i in the stimulation of MV release. Using the 17IIA11 (17A) cell line, we determined that P_i was responsible for the increased MV production (Figure 2A.). We also determined that the EV collected from the ECM possibly contained amorphous hydroxyapatite suggesting they are mineralization competent (Chaudhary, Kuzynski et al. 2016). Furthermore, when we exposed a vascular smooth muscle cell line (MOVAS) to elevated P_i, we observed an increase in MV release after 72h(Figure 2B) (Chaudhary, Khalid et al. 2018). These two studies confirmed the importance of P_i for induction of mineralization and led to new questions about how these MV are produced, what their function may be, and how P_i alters them.

Once we established that P_i induces MV release, we wanted to investigate whether the protein composition of the MV released from both 17A and MOVAS cells was altered due to P_i. We did this because it is thought that the composition of EV (of which MV is a subset) is thought to reflect their function. From our literature review, we also found that previous studies collected MV from both the medium and ECM of cultured cells for evaluation. However, we hypothesized that these vesicle populations would have different functional roles from each other and therefore varied compositions. As a result, we decided to evaluate them separately. Using mass spectrometric analysis, we determined that elevated Pi exposure alters the protein composition of EV from both the medium and ECM in 17A (unpublished, Figure 3A) and MOVAS cells (Figure 3B) (Chaudhary, Khalid et al. 2018).

From our previous findings, we hypothesized that elevated P_i alters the composition of MV through alteration of the gene expression of odontoblasts.
Figure 2. Phosphate induces extracellular vesicle release from mineralization-competent cells.

A. Comparison of the number of MV released by cells within 24h of stimulation with standard osteogenic medium (ascorbic acid and P_i; AA + P_i), 10 mM Na-Pi buffer (P_i), or ascorbic acid (AA), with cells cultured in standard growth medium (Cntrl). Data are represented as the mean values of three independent experiments ± SD, *p<0.05 and **p<0.005. B. Results of nanoparticle tracking analyses show the number of EVs isolated from conditioned medium (medium) or extracellular matrix (ECM) of MOVAS cells grown in the presence of 10 mM P_i for 24, 48 and 72h. Data are represented as the mean values of three independent experiments ± SD, *p<0.01. Adapted from (Chaudhary, Kuzynski et al. 2016)
Figure 3 Phosphate alters protein composition of EV from 17A and MOVAS cells

Summary of proteomic analyses of EVs released from MOVAS cells under standard growth conditions (Ctrl) and upon 24 h treatment with 10 mM Pi (Pi). EVs were isolated either from conditioned medium (medium) or from the extracellular matrix (ECM). A. Venn diagrams summarizing the effect of high Pi on EV protein composition of EV released from 17IIA11 (17A) cells B. Venn diagrams summarizing the effect of high Pi on EV protein composition released from MOVAS cells. Adapted from (Chaudhary, Khalid et al. 2018).
2.0 OBJECTIVE OF STUDY

Matrix vesicle (MV) release is odontoblasts has been under-studied. Most studies evaluate MV in the context of later stages of chondrogenesis or osteogenesis and without identification of the factor that stimulates the release of these vesicles. In our previous study, we identified Pi as the driver of an early MV release from a pre-odontoblast cell line and vascular smooth muscle cell line. Furthermore, we determined that elevated Pi exposure alters both the vesicle composition and the gene expression of osteogenic cells. Considering these observations, the objective of this study is to determine if alteration of gene expression is a mechanism through which vesicle composition is altered.
3.0 MATERIALS AND METHODS

3.1 CELL LINES

The 17IIA11 (17A) is a mouse pre-odontoblast cell line generated by Priam et al (2005). 17A were maintained in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 µg/ml streptomycin. This cell line was maintained at 37°C and at 5% carbon dioxide.

The MOVAS cell line is a mouse aortic smooth muscle cell line obtained from the ATCC. It was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA). This cell line was maintained at 37 °C and at 5% carbon dioxide.

3.2 EXPERIMENTAL DESIGN

3.2.1 Exome-Depleted Fetal Bovine Serum Preparation

FBS was diluted to a concentration of 20% with DMEM supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA). The FBS solution was centrifuged at 100,000 rpm in an ultracentrifuge for 14h to deplete vesicles from the FBS. The
FBS was then filter sterilized using Nalgene™ Rapid-Flow™ with 0.2 µm pores (Thery, Amigorena et al. 2006).

### 3.2.2 Experimental Conditions

17A and MOVAS cells were plated at 10 million cells per 10 cm dish in exome-depleted medium for 24 to 48h before treatment. DMEM was supplemented with exome-depleted FBS to a final concentration of 10% FBS exome-depleted medium for the 17A cell line and 20% FBS exome-depleted medium for the MOVAS cell line. High FBS concentrations were used to account for FBS loss during the exome depletion process.

For the experimental conditions, the medium was then further supplemented with 10mM sodium phosphate buffer (NaPi), the P\textsubscript{i} source (pH 7.4) (NaH\textsubscript{2}PO\textsubscript{4}•H\textsubscript{2}O and Na\textsubscript{2}HPO\textsubscript{4}) (as it has been shown that NaPi is a more controlled and direct source of P\textsubscript{i}) or ultra-pure water. The cells were then grown for 24h, 48h, and 72h for vesicle collection. After 48h the medium was replenished. Medium and ECM derived vesicles were then collected from the 24h group and from the ECM only at 48h and 72h groups. In all other experiments, 5% FBS (for 17A cells) and 10% FBS (for MOVAS cells) was used in place of exome-depleted FBS.
3.3 ISOLATION OF VESICLES

3.3.1 Medium-derived matrix vesicles

The medium MV was collected from the culture dish and centrifuged at 2,000g for 20min; the supernatant was then ready for further purification or stored in a 4ºC fridge for up to a week or in a -80ºC fridge indefinitely.

3.3.2 Matrix-derived extracellular vesicles

Once the medium was removed from the culture plate, the vesicles were released from the extracellular matrix (ECM) using enzymatic digestion. Specifically, For 17A cells DMEM was supplemented with 2.5 mg/ml collagenase IA (Sigma, St Louis, MO) and 2mM calcium chloride and added to the culture plates for 2h at 37ºC and 5% carbon dioxide for 17A cells. For MOVAS cells, 1 mg/ml of collagenase IA was used, and elastase (0.8U/ml) was also added to release matrix vesicles from MOVAS ECM.

Each mixture was then centrifuged for 20 min at 2000g, and the supernatant was immediately ready for further purification or storage in a 4ºC fridge for up to a week or in an 80ºC fridge indefinitely.
3.4 PURIFICATION OF VESICLES

All vesicles were purified using several steps of ultracentrifugation. First, the Medium and ECM collections were centrifuged at 4°C 10,000g for 30min to remove any cellular debris. The resulting supernatant was then transferred to clean tubes and centrifuged at 4°C at 100,000g for 1.5h to 3h. Next, the supernatant itself was discarded, and the pellet remaining after centrifuging was resuspended in PBS or Tris-buffered saline (TBS) (50 mM Tris–HCl, pH 7.4 and 154 mM NaCl) and centrifuged at 4°C at 100,000g for 70 min. The supernatant was again removed, and the remaining pellet was dissolved in PBS or TBS for further analysis and stored at -80°C (Thery, Amigorena et al. 2006).

The protein concentration of the vesicles was evaluated using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), and its color quantification was evaluated using a Synergy™ H1 microplate reader (BioTek, Vermont, USA).

3.5 QUALITATIVE AND QUANTITATIVE EVALUATION OF THE PREPARATION QUALITY OF EXTRACELLULAR VESICLES

3.5.1 Cryo-electron microscopy

Vesicles suspended in TBS were applied to holey carbon film (Quantifoil Micro Tools) and vitrified in liquid ethane using an FEI Vitrobot Mark IV. The grids were transferred to a Gatan 626 cryo-sample holder and imaged in an FEI Tecnai F20 electron microscope operated at 200
kV and nominal magnifications ranging from 32,750x to 65,500x. Low-dose images were captured with a Gatan Ultrascan 4000 CCD camera.

### 3.5.2 Nanoparticle tracking analysis

The size and concentration of the purified vesicles were determined by Nanoparticle Tracking Analysis (NTA) using NanoSight NS300 (Malvern Instruments Ltd, Worcestershire, UK). Data acquisition and analysis were performed using NTA 2.3 Analytical Software. One video file of 60 seconds at camera level 10 from each sample was created. A detection threshold limit of 10 was used to analyze the size and concentration of the vesicles.

The percentage of the vesicles in the size range of interest was determined by adding the number of particles within the size range of interest and dividing by the total number of particles.

### 3.5.3 Silver staining of proteins

Samples were prepared using 1x NuPAGE™ LDS buffer and 1x NuPAGE™ Sample reducing agent (Thermo Fisher Scientific). They were boiled for 10 min and incubated on ice for 5 min. These samples were also used for the western blot analysis. The vesicle proteins (5µg) were separated by SDS-PAGE electrophoresis using NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo Fisher Scientific) at 200V for 1h.

A Pierce™ Silver Stain Kit was used as instructed to stain the gel. A new sliver stain was done for each new vesicle preparation.
3.5.4 Western blotting

After the vesicle samples were prepared as described in the silver staining section, the vesicle proteins (7.5µg) were separated by SDS-PAGE electrophoresis using NuPAGE™ 4-12% Bis-Tris Protein Gels and transferred onto a nitrocellulose membrane. Specific proteins were detected with primary antibodies against: TNAP (1:1000; R&D Systems), GLG1 (1:1000; Sigma), OPN (1:10000; R&D Systems), and PIAD3 (1:1000 Proteintech). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences, Pittsburgh, PA, USA). All secondary antibodies (GE Healthcare or Fisher) were used at 1:3000 dilution (ani-rabbit, anti-rat, and anti-goat).

3.6 DETERMINING WHETHER PHOSPHATE-INDUCED GENE EXPRESSION CHANGES IN 17A CELLS CORRELATE WITH AN ALTERATION IN MV COMPOSITION

3.6.1 RNA isolation from total lysate of 17A cells

Total RNA was extracted using a GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St. Louis, MO, USA). The total RNA (1 µg), after DNase I treatment (Life Technologies, Grand Island, NY, USA), was converted to cDNA using a SuperScript III Reverse Transcriptase Kit (Life Technologies, Grand Island, NY, USA) and diluted in a ratio of 1:10 with ultra-pure water for further analysis.
3.6.2 **Reverse transcription polymerase chain reaction**

Gene expression analyses were performed using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) and Fast SYBR Green reaction mix (Roche, Indianapolis, IN, USA).

The primer sequences were (F denotes forward primer, R denotes reverse primer):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akp2</td>
<td>CAGTGGGAGTGAGCGCAGCC</td>
<td>GCACCTGGGTGTGGCGTGGTT</td>
</tr>
<tr>
<td>Dmp1</td>
<td>GCTGGGTCAACCACCACCACC</td>
<td>GGAGGGTCCTCCCCACTGTCC</td>
</tr>
<tr>
<td>Opn</td>
<td>ATGAGGCTGACCTCTCCTCTG</td>
<td>AAAGCTTCTTCTCTCTGAGCTGCCC</td>
</tr>
<tr>
<td>Ank</td>
<td>CACCCTGATAGCCTACAGTGAC</td>
<td>GGAAGGCAGCGAGTACAGG</td>
</tr>
<tr>
<td>Phospho1</td>
<td>ATGAGCGGGGTGTCTTCCAGC</td>
<td>GGTCTTCAACACATCTTCAGC</td>
</tr>
<tr>
<td>mPanx3</td>
<td>ACCCCCATGTCTTCTCGGAT</td>
<td>GTAGGTGAGCCAACATAGCCAAT</td>
</tr>
<tr>
<td>PTHR1</td>
<td>ACTGTGGCGATCCAGATGC</td>
<td>AATCTCTGCGTGCACCTCAC</td>
</tr>
<tr>
<td>Slc13a5</td>
<td>GTGGCGACTTTCAATCCA</td>
<td>TTGGGCGACTTTCCAATCCA</td>
</tr>
<tr>
<td>Itga6</td>
<td>CACTCAGGTTCGAGTGACGG</td>
<td>CAGCCTTGCTAGGTGTCAT</td>
</tr>
<tr>
<td>Cdsn</td>
<td>AATGTCCAGCCGCCCATAA</td>
<td></td>
</tr>
</tbody>
</table>
Expression was normalized to GAPDH expression, and relative expression was determined through comparison to the average control at the corresponding time point (ex: 48h control was compared to 48h Pi).

3.6.3 Statistical analysis

The vesicles were collected and pooled from ten 10cm dishes. RNA was collected from 3 controls, and 3 Pi treated 17IIA11 cells. Data are presented as the mean +/- standard deviation. Probability values were calculated using a Two-Tailed t-test, where \( p \leq 0.05 \) (*), \( p \leq 0.01 \) (**), and \( p \leq 0.001 \) (***) were considered to be statistically significant.
4.0 RESULTS

4.1 QUALITATIVE AND QUANTITATIVE EVALUATION OF THE PREPARATION QUALITY OF EXTRACELLULAR VESICLES

4.1.1 Shape of vesicles and quality of preparation based on Cryo-EM

4.1.1.1 Shape and quality of extracellular vesicles collected from MOVAS cells

EV are spherical membrane-enclosed particles. For our study, we were interested in vesicles ranging in size from 50nm to 220nm, as this size range contains MV (Chaudhary, Kuzynski et al. 2016, Chaudhary, Khalid et al. 2018). To evaluate the purity of the vesicles collected during the process described above, cryo-electron microscopy was performed on MOVAS cells.

Vesicles were collected from MOVAS cells from either the medium or ECM and after elevated Pi exposure for 24h. Four groups were evaluated: Control EV-Medium, Pi EV-Medium, Control EV-ECM, and Pi EV-ECM. The Cryo-EM images showcased in Figure 4 demonstrate vesicles within the size range of approximately 50 to 120nm (indicated by white arrows). From the images, we can conclude that the vesicles collected using the ultracentrifugation method reside within the MV range of interest (50nm to 220nm) and are relatively pure, as indicated by the lack of significant contamination by other particles. This is especially true of the ECM groups, as ECM contamination by ECM collagen fibrils is absent.
Figure 4. Cryo-EM of extracellular vesicles (EV) released by MOVAS cells

Cells were grown in standard conditions (Ctrl) or with 10mM phosphate (P.). Vesicles were collected from ECM and medium after 24h. Scale bars are 100nm. Adapted from (Chaudhary, Khalid et al. 2018).
4.1.2 Verification of mass spectrometric analysis

Further analysis of the proteins detected by mass spectrometry was done to validate the mass spectrometric analysis of the 17A cells, which was only performed once. To determine the quality of this analysis, we attempted to evaluate several proteins that were detected in MV using mass spectrometric analysis by using a western blot analysis on new samples. We evaluated the same four groups used for mass spectrometry analysis (Control MV-Medium (Crtl-med), Phosphate MV-Medium (P$_i$-med), Control MV-ECM (Ctrl-ECM), Phosphate MV-ECM (P$_i$-ECM)). Of all the proteins detected, we chose proteins based on their detection from the mass spectrometric analysis. For example, some proteins were present in all samples, some only in the ECM group (ECM), some only in the medium (med) group; meanwhile, other proteins showed significant alterations by phosphate, both increase and decrease presence in vesicles. Unfortunately, of the 14 proteins tested, only 2 were detectable by western blot. This could be due to poor antibodies or an insufficient amount of protein present to be detectable.

Golgi Glycoprotein 1 (Glg1) and Protein Disulfide Isomerase Family A Member 3 (Piad3) were the two proteins detectable by western blot. The mass spectrometric data showed a significant increase by elevated P$_i$ in the ECM group, and we saw the same pattern using western blot analysis (Figure 5A). Silver staining of gels using the same vesicle preparation was used as a loading control. Of note from the silver stain is the different banding pattern in the vesicles collected from the medium and ECM (Figure 5B). This further confirms, after mass spectrometric analysis, that the MV collected from the medium and ECM are different vesicle populations.
Figure 5. Western blot analysis of proteins in MV released by 17IIA11 cells into the medium (med) or ECM after 24h of elevated phosphate exposure.

A. Western blot of MV detecting Pdia3 and Glg1. 7.5ug of protein/lane. B. Silver stain of gel. 5ug of protein/lane. This does not seem to be correct formatting – please check.
4.1.3 Size distribution analysis of ECM vesicles based on nanoparticle tracking analysis

From the mass spectrometric analysis and the silver staining of MV proteins, we determined that the vesicles isolated from the ECM are significantly different from those isolated from the medium. Next, we focused on the ECM-derived vesicles to determine the role of phosphate in MV biogenesis. Vesicle biogenesis pathway effects the size of the EV released, the biogenesis pathway for exosomes, which are formed in the cell and released through exocytosis, results in vesicles that range in size from approximately 50nm to 80nm. On the other hand, apoptotic bodies, which are released during apoptosis, range in size from approximately 500nm to 1µm (Yuana, Sturk et al. 2013, Yanez-Mo, Siljander et al. 2015, Zaborowski, Balaj et al. 2015, Bottini, Mebarek et al. 2018). As vesicle isolation results in a heterogeneous population of vesicles, it is important to verify if the isolation method results in the collection of MV. If size distribution is altered, it may be due to a change in the biogenesis pathway. We evaluated the MV released from 17A cells into the ECM over 72h after exposure to 10mM Pi. This resulted in 6 groups being used for further study: 24H Control ECM MV (24H C ECM), 24H Pi ECM MV (24H P ECM), 48H Control ECM MV (48H C ECM), 48H Pi ECM MV (48H P ECM), 72H Control ECM MV (72H C ECM), and 72H Pi ECM MV (72H P ECM). We assessed the size distribution of MV using nanoparticle tracking analysis to determine if Pi alters vesicle size distribution.

4.1.3.1 Evaluating extracellular vesicle preparation using nanoparticle tracking analysis

The vesicle size range of interest to this study is between 50nm to 220nm, which includes exosomes (50nm to 79nm in size) and matrix vesicles (80 nm to 220 nm in size). For the purpose of our study, we strictly classified the vesicles collected based on size; it should be noted that
these are the approximate size ranges of exosomes and MV respectively. Particles smaller than 50nm are most likely small protein aggregates or small pieces of cellular debris. Larger particles may be protein clumps, large pieces of cellular debris that weren’t removed, or apoptotic vesicles.

Our vesicle preparations were between 73% and 88% vesicles of interest (Table 1.) This is similar to preparations done in our lab previously (Chaudhary, Kuzynski et al. 2016).
Table 1. Nanoparticle Tracking Analysis of MV released from 17IIA11 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Sample Size</th>
<th>Mode Sample Size</th>
<th>Percent of Sample Sized between 50 and 80nm (Exosomes)</th>
<th>Percent of Sample Sized Between 80 and 220nm (Matrix Vesicles)</th>
<th>Total Preparation in Range of Interest (50nm to 220nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 H C ECM</td>
<td>96nm</td>
<td>59nm</td>
<td>28%</td>
<td>47%</td>
<td>75%</td>
</tr>
<tr>
<td>24H P ECM</td>
<td>97nm</td>
<td>75nm</td>
<td>25%</td>
<td>53%</td>
<td>78%</td>
</tr>
<tr>
<td>48H C ECM</td>
<td>129nm</td>
<td>104nm</td>
<td>18%</td>
<td>70%</td>
<td>88%</td>
</tr>
<tr>
<td>48H P ECM</td>
<td>121nm</td>
<td>71nm</td>
<td>24%</td>
<td>59%</td>
<td>83%</td>
</tr>
<tr>
<td>72H C ECM</td>
<td>127nm</td>
<td>70nm</td>
<td>18%</td>
<td>55%</td>
<td>73%</td>
</tr>
<tr>
<td>72H P ECM</td>
<td>145nm</td>
<td>89nm</td>
<td>16%</td>
<td>64%</td>
<td>80%</td>
</tr>
</tbody>
</table>
Figure 6. Size distribution of EV collected from 17HA11 cells.

Cells were grown under standard growth conditions (Ctrl) or with 10 mM Pi (Pi). EVs were isolated from the extracellular matrix (ECM) after 24, 48, and 72h. Vesicle preparations were evaluated using nanoparticle tracking analysis. The graphs demonstrate the size distribution of particles contained within the samples. Again – check to see how much info should be here under the figure.
4.1.3.2 Comparing extracellular vesicles released from 17IIA11 cells grown in elevated Phosphate Conditions

Although P_i exposure has been shown to alter the composition of MV, the mechanism through which this alteration occurs is unknown. One-way P_i may alter vesicle composition is through alteration of vesicle biogenesis. We used nanoparticle tracking analysis to determine if the size of the vesicles changed in response to elevated P_i. Using the nanoparticle tracking data, we evaluated the size distribution of MV between treated and non-treated groups at the same time point.

We did not identify differences in size distribution between the control and P_i groups at each time point. Comparing the mean particle size of control at and P_i groups at 24h, we found that it was 96 and 97nm, respectively. The mean particle size of control at 48h and P_i at 48h was 129 and 121nm, respectively. A larger difference in mean particle size was observed between the control at 72h and P_i at 72h groups, 127 and 145nm, respectively, however, this difference is due to increased contamination by particles larger than 220nm, not due to an increase in average vesicle size (Table 1 and Figure 6).

4.1.3.3 Comparing extracellular vesicles released from 17IIA11 cells grown in elevated phosphate conditions over time

To determine if MV size changed over time in response to elevated P_i, we used the nanoparticle tracking data to analyze the size distribution of vesicles released by 17A cells over time, from 24h of elevated P_i exposure to 72h of elevated P_i exposure. We did not identify differences in size distribution within the vesicle groups collected from the ECM over time.

In the 24h P_i group, the mean particle size was 97nm, and the mode size of the particles detected was 75. After 48h the mean particle size increased to 121nm, but the mode particle size was only
71nm. Further, after 72h, the mean particle size increased to 145nm, but the mode particle size only increased to 89. These changes are in the lower range of MV size and are not meaningfully different. However, as this experiment was only performed once, statistical significance could not be ascertained. (Table 1 and Figure 6). The increasing mean identified here can be explained by an increase in the number of contaminating particles over 220nm the longer treatment continues. The mode particle size of each sample is the size of particle most commonly detected. This does not shift meaningfully as the experiment progresses. This indicates that the pathway producing the vesicles is still the same, even though the mean is increasing.

4.2 DETERMINING WHETHER PHOSPHATE-INDUCED GENE EXPRESSION CHANGES IN 17A CELLS CORRELATE TO AN ALTERATION IN MV COMPOSITION

In an unpublished RNA-seq analysis, we determined that elevated Pi alters the gene expression of 17A cells. Many of the genes affected are known to be involved in the mineralization of the ECM (TNAP, Dmp1, and Opn). Pi also induces the release of MV, the nucleation sites of hydroxyapatite (Chaudhary, Kuzynski et al. 2016). As we have shown that exposure to elevated Pi alters the composition of MV, we wanted to determine if some of the alterations in MV composition we identified were due to the alteration of gene expression.
Genes were chosen for examination based on at least one of four criteria (genes selected based on the criteria stated are listed in ()): alteration in expression in response to Pi as indicated by RNA-seq analyses (all genes selected for analysis), presence in MV as indicated by our mass spectrometry analysis (Dmp1, Opn, Itag6, Akp2, Pth1r, Panx3, Slc13a5, Cdsn), presence in EV based on the literature (Itga6, Sema7a, TNAP, Panx3, and Slc13a5 (Thouverey, Malinowska et al. 2011)), or function (all genes selected for analysis).

4.2.1 Determination if Pi induced MV composition change is related to Pi-induced gene expression changes (increased expression).

Osteopontin (Opn) is a non-collagenous protein present in the extracellular matrix (ECM) of mineralized tissue (McKee and Nanci 1996). Elevated Pi significantly increases the expression of Opn in 17A cells after 24h (p ≤ 0.001), and increased expression was continued for 72h (p ≤ 0.05) (Figure 7). A small number of particles of Opn were detected in the medium and the ECM vesicles after exposure to Pi by mass spectrometry analysis, but this was not replicated in the western blot analysis (Figure 8, and Table 2). The detected levels of Opn in the mass spectrometry analysis were at low levels, which may not have been sufficient for detection by western blot. There was also a significant amount of background, which may have obscured the signal.

Progressive ankylosis protein (Ank) is a transmembrane protein that transports inorganic pyrophosphate from the intercellular space into the ECM. Loss of this protein in mice has been shown to decreased bone mass (Kim, Minashima et al. 2010, Wuthier and Lipscomb 2011). In
our study, *Ank* showed a significant increase in gene expression at 24h (p ≤ 0.001) and 48h (p ≤ 0.05) after P$_i$ exposure (Figure 7). *Ank* was not detected in the matrix vesicles (Table 2).

Dentin matrix phosphoprotein 1 (*Dmp1*) is another protein that localizes to the ECM; it is responsible for nucleating hydroxyapatite (Wu, Teng et al. 2011). Elevated P$_i$ significantly increased expression of *Dmp1* in 17A cells after 24h (p ≤ 0.001), and this increased expression was maintained for 72h (p ≤ 0.05) (Figure 7). A small number of particles of Dmp1 were detected in medium vesicles after exposure to P$_i$ (Table 2).

Integrin alpha 6 (*Itga6*) is a cell-surface adhesion receptor that binds to the ECM and mediates cell–ECM interactions (Wang, Choi et al. 2014, Yanez-Mo, Siljander et al. 2015). Elevated P$_i$ significantly increased the expression of *Itga6* in the 17A cells after 24h (p ≤ 0.001), and the increased expression was maintained for 48h (p ≤ 0.05) (Figure 7). It was detected in the vesicle groups: Cntrl-Medium EV, P$_i$–Medium EV, and P$_i$–ECM MV, but there was no significant alteration by P$_i$ (Table 2).

Semaphorin 7A (*Sema7a*) is a GPI-anchored membrane glycoprotein, and mutations of this gene have been implicated in reduced bone mineral density (Delorme, Saltel et al. 2005). *Sema7a* was not detected in matrix vesicles, even though it showed a significant increase in expression after 24h (p ≤ 0.01) and maintained the significant increase in expression at 72h (p ≤ 0.05) (Figure 7 and Table 2).
Dual Specificity Phosphatase 6 (Dusp6) is a cytosolic MAP kinase phosphatase that specifically recognizes and inactivates the ERK1/2, a known mediator of P1 signaling. In a previous study, a Dusp6 mutant mouse showed dwarfism and coronal craniosynostosis (Bradshaw and Dennis 2010). Dusp6 was not detected in the matrix vesicles, even though it showed a significant increase in expression after 24h (p ≤ 0.01) and maintained increased expression for 48h (p ≤ 0.05) (Figure 7 and Table 2).
Figure 7. Analysis of upregulated genes in 17A cells after exposure to elevated P<sub>i</sub>

RNA from 17IIA11 cells were collected under standard growth conditions (Control) at 24h, 48h, 72h and upon 24h, 48h, and 72h after treatment with 10 mM P<sub>i</sub> (P<sub>i</sub>). Fold change for the phosphate treated groups was determined relative to control, which was set at 1. p ≤ 0.05 (*), p ≤ 0.01 (**), and p ≤ 0.001 (***). p ≤ 0.05 (*), p ≤ 0.01 (**), and p ≤ 0.001 (***).
Table 2. Detection pattern in MV of genes upregulated by Pi using mass spectrometry

<table>
<thead>
<tr>
<th>Gene</th>
<th>Detection of Protein in MV after 24h Pi exposure (17A)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dmp1</em></td>
<td>P$_i$ –Medium EV</td>
</tr>
<tr>
<td><em>Ank</em></td>
<td>No detection</td>
</tr>
</tbody>
</table>
| *Opn* | P$_i$ –Medium EV  
P$_i$ –ECM MV |
| *Itag6* | Cntrl-Medium EV  
P$_i$ –Medium EV  
P$_i$ –ECM MV |
| *Sema7a* | No detection |
| *Dusp6* | No detection |
Figure 8. Western blot analysis of proteins in MV released into the ECM from 17HA11 cells after 72h of elevated P, exposure.

A. Western blot analysis of Opn and Akp2/TNAP. MV loaded at 7.5ug/lane and total cell lysate at 10ug protein/lane. B. Silver stained gel of MV from the same preparation as those prepared for western blot 5ug protein/lane were loaded.
4.2.2 Determination if Pi induced MV composition change is related to Pi-induced gene expression changes (decreased expression).

Tissue non-specific alkaline phosphatase (*Akp2/TNAP*) is a membrane protein that converts pyrophosphate to Pi. Mutations in *AKP2* lead to a condition called hypophosphatasia, where bone mineralization is severely impaired (Ciancaglini, Yadav et al. 2010, Wuthier and Lipscomb 2011, Huesa, Houston et al. 2015). At 24h, 48h and 72h of elevated Pi exposure, Akp2 showed a significant decrease in expression using qRT-PCR (*p* ≤ 0.05, *p* ≤ 0.01, and *p* ≤ 0.05, respectively) (Figure 9). Akp2 was detected in all vesicle groups, but no significant change in Akp2/TNAP was detected by mass spectrometry or western blot (Figure 8 and Table 3).

Phosphoethanolamine/Phosphocholine Phosphatase (*Phospho1*) is a member of the haloacid dehalogenase superfamily. It is involved in MV-mediated mineralization. It plays a role in savaging Pi within the MV lumen to promote hydroxyapatite formation. Phospho−/− mice have shown decreased mineral density among other skeletal abnormalities (Roberts, Stewart et al. 2004, Ciancaglini, Yadav et al. 2010, Huesa, Houston et al. 2015). Over 24h and 48h, *Phospho1* showed a significant decrease in expression using qRT-PCR (*p* ≤ 0.05, and *p* ≤ 0.01, respectively) (Figure 9). It was not detected in MV (Table 3). This shows that it was never trafficked into the MV and, therefore, its decreased expression does not affect MV composition.

Parathyroid hormone 1 receptor (*Pth1r*) is the receptor for the parathyroid hormone. It is a transmembrane protein that is part of the regulatory pathway that maintains phosphate and calcium homeostasis. Several human diseases with bone and dental phenotypes are associated with mutations in *PTH1R*, such as Blomstrand’s lethal chondroplasia, and Familial primary failure of tooth eruption (Cheloha, Gellman et al. 2015, Fan, Bi et al. 2016). *Pth1r* showed a...
significant decrease in expression after 24h (p ≤ 0.01) and 48h (p ≤ 0.05) (Figure 9). It was only detected in the control vesicles at a low level (Table 3).

Pannexin 3 (Panx3) is a protein involved in cell-cell and cell-matrix communication. Mice lacking Panx3 have shown delayed and disorganized mineral deposition (Oh, Shin et al. 2015, Ishikawa and Yamada 2017). After 24h of P_i exposure, Panx3 showed a significant decrease in expression (p≤ 0.001) as well as at 48h (p ≤ 0.05) and 72h (p ≤ 0.01) using qRT-PCR (Figure 9). It was present in all 17A released extracellular vesicles (EV) and showed no significant alteration by P_i (Table 3).

Solute Carrier Family 13 Member 5 (Slc13a5) is a sodium-dependent citrate transporter. Up to 70% of citrate is stored in bone, and citrate helps stabilize and nucleate hydroxyapatite crystals. Slc13a5^-/- C57BL/6 mice have shown decreased bone mineral density and impaired bone formation, along with impaired enamel formation (Irizarry, Yan et al. 2017). Slc13a5 showed a significant decrease in expression over 24h (p ≤ 0.01) and 48h (p ≤ 0.01) using qRT-PCR (Figure 9). Slc13a was present at a low level in the control vesicle groups but not in the vesicles treated with Pi (Table 3).

Corneodesmosin (Cdsn) is an extracellular matrix/cell adhesion molecule (Xiao, Camalier et al. 2007). After 24h of P_i exposure, it showed a significant decrease in expression (p ≤ 0.05) as well as at 48h (p ≤ 0.01) and 72h (p ≤ 0.05) using qRT-PCR (Figure 9). In the mass spectrometry analysis, it was only detected in the P_i-MV group, but at low levels (Table 3). This finding is contrary to the gene expression analysis, indicating that the decrease in Cdsn mRNA does not affect its trafficking into the MV.
In summary, when gene expression changes are compared to MV composition, the changes do not appear to correlate to MV composition after 24h of P_i exposure. Furthermore, analysis by western blot of Opn and TNAP in MV does not show a correlation to gene expression.
RNA from 17IIA11 cells were collected under standard growth conditions (Control) at 24h, 48h, 72h and at 24h, 48h, and 72h after treatment with 10 mM P_i (P_i). Fold change for the phosphate treated groups was determined relative to control, which was set at 1 p ≤ 0.05 (*), p ≤ 0.01 (**), and p ≤ 0.001 (***)

Figure 9 Analysis of downregulated genes in 17A cells after exposure to elevated Pi
Table 3. Detection Pattern in MV of Genes Downregulated by Pi Using Mass Spectrometry

<table>
<thead>
<tr>
<th>Gene</th>
<th>Detection in EV after 24h Pi exposure (17A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akp2</td>
<td>Present in all samples. <em>No significant alteration by Pi</em></td>
</tr>
<tr>
<td>Phospho1</td>
<td>No detection</td>
</tr>
<tr>
<td>Pth1r</td>
<td>Cntrl –Medium EV Cntrl–ECM MV</td>
</tr>
<tr>
<td>Panx3</td>
<td>Present in all samples. <em>No significant alteration by Pi</em></td>
</tr>
<tr>
<td>Slc13a5</td>
<td>Cntrl –Medium EV Cntrl–ECM MV</td>
</tr>
<tr>
<td>Cdsn</td>
<td>Pi –ECM MV</td>
</tr>
</tbody>
</table>
5.0 DISCUSSION

Recently, our lab has established that exposure to elevated $P_i$ induces the release of matrix vesicles (MV) from both pre-odontoblast and vascular smooth muscle cells (Chaudhary, Kuzynski et al. 2016, Chaudhary, Khalid et al. 2018). Using mass spectrometric analysis, we determined that exposure to elevated $P_i$ alters the composition of these vesicles (Figure 2) (Chaudhary, Khalid et al. 2018). In an unpublished RNA-seq analysis evaluating the effect of $P_i$ on pre-odontoblast cells, we identified gene expression changes of many proteins involved in mineralization. The purpose of the present study was to determine if gene expression changes induced by elevated $P_i$ contributed to alteration of MV composition.

While not the focus of the present investigation, we determined that $P_i$ did not alter the size distribution of MV released from 17A cells. As size distributions are indications of vesicle type and therefore biogenesis pathway, this finding suggests that vesicles are generated through the same pathway even though their composition, and presumably function, are altered.

This study is the first to evaluate the relationship between gene expression and MV composition. We demonstrated significant changes in gene expression when 17A cells were exposed to elevated $P_i$ over 72h. However, this did not appear to correlate with changes in the protein composition of the MVs (Figure 7, 8, 9, Table 2 and 3). This suggests that phosphate signaling, especially at the 24h time point, regulates protein processing and/or trafficking, which will direct relevant proteins into MV, separate from its action on gene expression.
However, this does not mean that gene expression changes do not contribute to the alteration of vesicle composition at later timepoints. Another possibility for why gene expression and alteration of protein composition do not show a correlation is that the timeframe used in this study to evaluate protein alteration may have been too short to determine the effects of gene expression changes. A longer, more comprehensive evaluation of MV composition and transcriptome analysis would need to be performed to determine if gene expression changes alter MV composition after longer exposure to elevated Pi.

Gene expression may also alter the composition of vesicles through alteration of the RNA composition of MV. EV are known to contain different types of RNA, including mRNA (György, Szabó et al. 2011, Yuana, Sturk et al. 2013, Zaborowski, Balaj et al. 2015, Rilla, Mustonen et al. 2017). Analysis of the proteins altered by Pi in MV released from predontoblasts and vascular smooth muscles cells identified proteins involved in RNA processing overrepresented in proteins increased by Pi in MV (Figure 10). The alteration of these RNA processing proteins by Pi in MV may also indicate a change in MV nucleic acid composition. A way to evaluate this possibility would be to evaluate the nucleic acid composition of MV and compare that to total cell gene expression.

The MV samples used to evaluate changes in vesicle composition were most likely a heterogeneous EV population. In order to determine the purity of our samples, we implemented several quality control experiments: Cryo-EM, silver stain, and nanoparticle tracking analysis. We have determined that this collection mostly contains MV because of the size distribution profile and enrichment of TNAP (a protein enriched in MV). However, the collection most likely contains a small population of other types of EV as well. Most published evaluations of EV employ differential centrifugation to concentrate and partially purify vesicles. Generally, the
centrifugation steps include depletion of cellular debris then isolation of vesicles using greater than 100,000g speeds. The centrifugation speed can be calibrated to collect EV within a size range of interest. Using this method, we collected vesicles between 50nm and 220nm, and we confirmed our collection method by evaluating MV samples collected from MOVAS cells using Cryo-EM. However, these samples are still heterogeneous in nature (Thery, Amigorena et al. 2006).

Another method of EV isolation is size-exclusion chromatography. In this method, the EV are isolated based on size through a filter column. However, this method requires a pre-concentration step that may result in deformed EV or their rupture. This method will also result in different types of EV being present in the sample if they are in the same size range (Szatanek, Baran et al. 2015). Due to the heterogeneous nature of the sample preparation, there is a possibility that subtypes of MV are responsible for distinct functions: some to form hydroxyapatite, some to alter the ECM, and others to facilitate cell-cell communication (Witwer, Buzás et al. 2013). New techniques will need to be developed to better isolate and evaluate MV to determine more about their biogenesis and range of functions.

In this study, we evaluated the protein MV released from pre-odontoblastic cells. Other studies, however, have evaluated MV protein composition using osteoblasts or chondrocytes in vitro and cartilage tissue from an in vivo model. One group, for example, collected MV from femurs of chicken embryos, and while they identified many of the same proteins found in mouse osteoblast and chondrocyte MVs, they had different amounts present, indicating that it is important to consider the source when evaluating composition (Balcerzak, Malinowska et al. 2008).
Other studies have focused only on the composition of MV released from differentiating precursor cells without evaluating how the composition of MV may change. Our previous studies have shown that elevated P\textsubscript{i} exposure not only stimulates MV release, but it also alters the composition of those MV. One group obtained MV from differentiating MC3T3-E1 cells and evaluated vesicles from both the ECM and the medium after 15 days of osteogenic differentiation. They identified many proteins involved in mineralization, such as TNAP. However, they did not fully address if there were differences between the medium and ECM groups. Instead, they assumed that the vesicles collected from both were the same (Xiao, Camalier et al. 2007). The novelty of our previous studies in comparison to both these proteomic approaches is that we attempted to, and did, identify a MV release initiator (P\textsubscript{i}). Using proteomic analysis, we were able to determine that vesicles released into the medium and ECM are different vesicles populations. Finally, we determined that P\textsubscript{i} alters the composition of MV. These findings led us to wonder how P\textsubscript{i} alters the composition of vesicles (Chaudhary, Kuzynski et al. 2016, Chaudhary, Khalid et al. 2018).

This present study is the first to evaluate a possible mechanism driving changes in MV composition released from osteogenic cells undergoing mineralization. By understanding the mechanism behind altered vesicle composition, we may be able to harness this understanding to develop new drug delivery or drug packaging methods. Future studies should employ a comprehensive and long-term evaluation of MV composition in comparison to gene expression to further elucidate a possible mechanism driving composition change and modulation of MV cargo.
Figure 10. Protein profiling of EV released from 17IIA11 cells and altered by elevated phosphate.

EV released from 17IIA11 cells were collected under standard growth conditions (Ctrl) and upon 24h treatment with 10 mM Pi (Pi) both from conditioned medium (Med) and from the extracellular matrix (ECM). Bar chart of difference presenting the statistical overrepresentation test analysis results of proteins significantly changed by Pi (>3-fold difference) and proteins present only in either Ctrl or in Pi group in annotation datasets of PANTHER GO-Slim for Biological process and molecular functions. All 1164 proteins identified by mass spectrometry were used as a reference. Statistically significant (p<0.05) results are shown.
6.0 CONCLUSIONS

In conclusion, this study demonstrated that while vesicle composition is altered due to $P_i$, MV size distribution is not altered. This indicates that the biogenesis pathway producing MV is unaltered by $P_i$, but the composition of the vesicles is affected. We attempted, for the first time, to determine a possible mechanism driving composition change in MV by $P_i$. From these studies, we have determined that gene expression changes are not responsible for changes in MV composition in the time frame evaluated (24h). This indicates that $P_i$ signaling most likely influences trafficking and/or packaging of proteins separate from its influence on gene expression.

However, this does not mean that RNA expression changes do not alter the composition of vesicles. First, the transcriptome of MV may be altered or a longer evaluation period may be needed to determine if vesicle composition correlates to gene expression changes. Further studies evaluating MV composition over time will be required to investigate these possibilities.
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