CHARACTERIZING AND COMPARING DENTAL CELL SECRETED EXOSOMAL VESICLES.

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ABSTRACT

Recent reports suggest promise for cell secreted extracellular vesicles (EVs) in regenerative medicine based on their roles as important mediators of intercellular communication and diverse physiological and pathological processes. EVs isolated from dental cells harvested from normal patients, and those with genetic diseases such as Fibrodysplasia Ossificans Progressiva (FOP), a rare genetic disease characterized by the progressive development of heterotopic ossification, may provide valuable insights into the underlying disease mechanisms, as well as new approaches to regenerate bone in an effective and controlled manner. **Objective**: The objective of this study is to isolate, characterize and compare EVs and their cargo secreted from three types of in vitro cultured dental stem cells: 1) Wild type (normal patient) dental pulp stem cells (DPSCs); 2) Wild type periodontal ligament stem cells (PDL cells); and 3) FOP patient derived DPSCs. **Materials and Methods**: EVs collected from replicate (3) in vitro cultured cells harvested by successive filtration using Centricon filters, Izon columns (Izon sciences, New Zealand) and Amicon filters (Millipore, Burlington, MA). EV yields and sizes were characterized by nanoparticle tracking analysis (Zetaview, Particle Metrix, Ammersee, Germany). Small RNA sequencing libraries were then constructed from EV RNA using QIASeq miRNA library kit (Qiagen, Hilden, Germany). Differential small RNA profiling was then used to define miRNA profiles of each dental cell type. Western blot analysis was conducted to study protein content. Statistical analyses include Welch's ANOVA, Independent sample t-test, Mann-Whitney U test and Kruskal- Wallis test. **Results**: Comparable EV yields were obtained from all dental cell lines. EV

particle size distribution was relatively uniform with an average diameter of approximately ~104.16nm ± 3.53nm. No statistical differences were observed in small RNA yields from dental cell EVs. Western blot analyses validated EV protein isolations. **Conclusions**: We have successfully isolated dental cell EVs. Comparable EV yields were obtained from all dental cell lines. miRNA isolations and profiling were successful for all dental cell lines. Ongoing analyses of EV cargo in each cell type will elucidate roles for dental EV signaling in health and disease.

***Keywords**:* Exosomal Vesicles (EVs), Micro RNA (miRNA), Fibrodysplasia Ossificans Progressiva (FOP), Osteogenic media (OM).

DEDICATION

I would like to dedicate my thesis to my parents who instilled in me the love of learning and the pursuit of knowledge.

To my family whose sacrifices and encouragement made this journey possible.

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ABBREVIATIONS

BCA assay: Bicinchoninic acid assay
BMP: Bone Morphogenetic Protein
BSA: Bovine Serum Albumin
cDNA: Complementary DNA
CMF: Craniomaxillofacial
DPSC: Dental Pulp Stem cell
EVs: Exosomal Vesicles
FOP: Fibrodysplasia Ossificans Progressiva
miRNA: Micro RNA
mRNA: Messenger RNA
Nm: nanometers
NM: Normal media
NTA: Nanoparticle tracking analysis
OM: Osteogenic media
PDLSC: Periodontal ligament stem cells
piRNA: Piwi interacting RNA
PVDF membrane: Polyvinylidene difluoride membrane
RIPA: Radioimmunoprecipitation assay buffer
RT: Room temperature
SEC: Size exclusion chromatography
siRNA: small interfering RNA
UTR: Untranslated region
WB: Western Blot
WT: Wild type

INTRODUCTION

Extracellular Vesicles (EVs) are small, cell-secreted, membrane bound vesicles that typically range from 40-150 nanometers (nm) produced by multivesicular bodies **(Figure 1**). (Kalluri and LeBleu 2020). EVs are secreted from both prokaryotic and eukaryotic cells (Shu Hua 2021). EVs can be classified into three major subtypes which include micro vesicles, exosomes and apoptotic bodies which show variations in size, morphology and content (Yonghee Song 2020) (microvesicles:50-1,000nm,exosomes:40-150nm and apoptotic bodies:1-5um). They exhibit a round shaped morphology under a scanning electron microscopy (Fatemeh Rahmatinejad 2024). They contain bioactive molecules including lipids, proteins, DNA, mRNA and non-coding RNAs such as microRNAs(miRNAs), Circular RNA (circRNA), PIWI-interacting RNAs (piRNAs) (Yu Jin Lee 2024). They were first found in sheep reticulocytes in 1983 (Yi Zhang 2020).

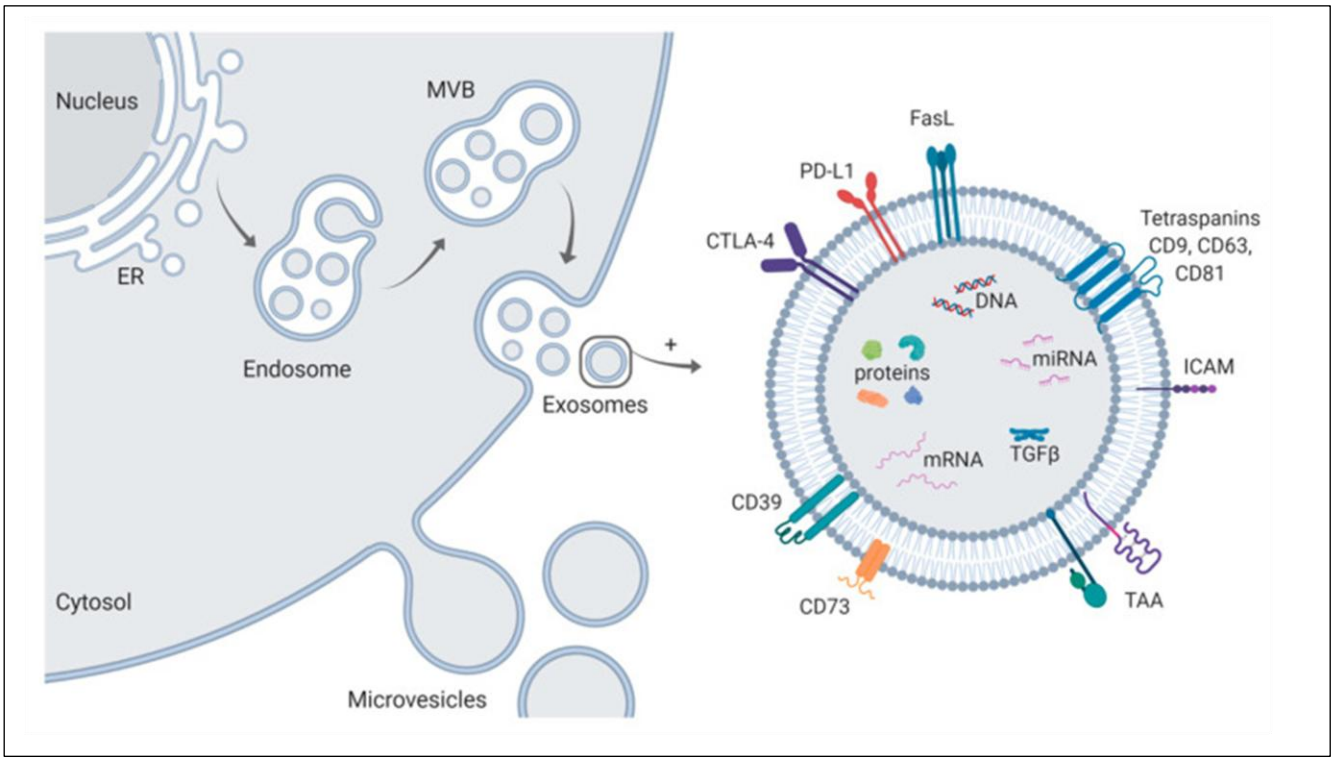


Figure 1. Schematic representation of the exosome biogenesis and molecular cargo. Adapted from (Hofmann et al. 2020)

They show promise for applications in regenerative medicine based on their roles as important mediators of intercellular communication and diverse physiological and pathological processes and affect various aspects of cell biology (Kalluri and LeBleu 2020). They represent a novel mode as intercellular communicators which play a major role in many cellular processes such as signal transduction, immune response, antigen presentation (Yuan Zhang 2019) by transferring cell proteins, lipids, nucleic acids, metabolites etc, which can be secreted under pathologic and normal conditions (Kalluri and LeBleu 2020).Due to their characteristics and biological functions they can be utilized as novel biological platform for diagnosis of disease and therapeutics (Yonghee Song 2020). They serve as potential biomarkers for diseases including cancer and are also being explored for their therapeutic applications such as drug delivery systems. Their ability to encapsulate and protect therapeutic agents makes them an exciting area of study for improving treatment efficacy and reducing the side effects. They assist in immune response modulation, removal of cellular debris and also enable waste management by packaging and exporting material that is damaged which includes misfolded proteins or toxins from the cell (Xing et al. 2020). They are released into extracellular matrix after fusion of the poly-vesicular outer membrane and the cell membrane so that the biological function of the exosomes is determined by the source cells (Kalluri and LeBleu 2020).

In the presence of pathogens, exosomes stimulate or inhibit the aspects of innate and adaptive immunity system and further allowing the pathogen to transition back to homeostasis (Kalluri and LeBleu 2020). They also participate in all cell interactions, especially antigen presentation, information transmission, target and immune cell regulation, tissue regeneration to name a few. Their natural ability to carry cargo makes them promising vehicles of targeted

therapies. The cargo which is carried by exosomes is crucial for their biological function and can also influence recipient cells in different ways. One of the roles of cargo in exosomes is molecular transport and communication. Their biologically active cargo may offer prognostic information in a range of diseases such as neurodegenerative diseases, cardiovascular and renal diseases, tumors, chronic inflammation (Yuan Zhang 2019). They are secreted by a wide variety of cells, including immune cells, cancer cells and stem cells like dental pulp stem cells (DPSC), periodontal ligament stem cells (PDLC) and Fibrodysplasia Ossificans Progressiva (FOP-DPSC) (Yi-Fan Chen 2024).

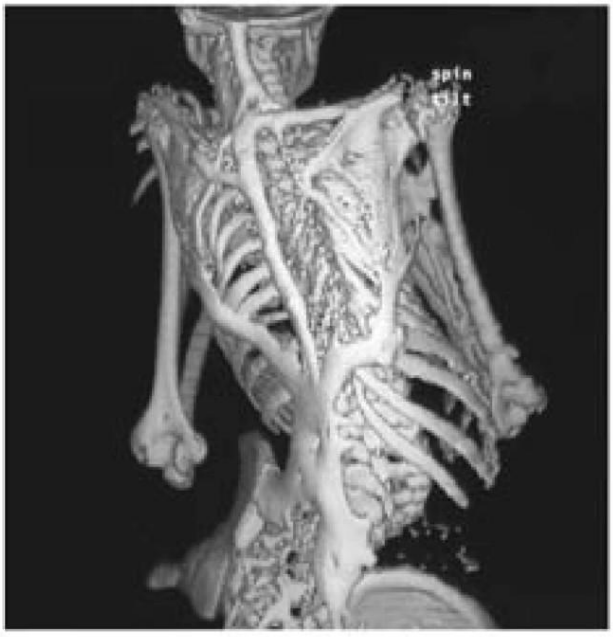


Figure 2. Three-dimensional reconstructed computed tomography (CT) scan of the back of a twelve-year old child showing extensive heterotopic ossification typical of FOP. Adapted from (Robert J Pignolo 2011).

Fibrodysplasia Ossificans Progressiva (FOP), is a rare autosomal dominant disorder characterized by heterotopic endochondral ossification at multiple sites, predominantly the muscles, ligaments, fascia and tendons. (**Figure 2**). It is caused by activating mutations seen in ACVR1/ALK2 **(Figure 3**) gene located over chromosome 2 and the involvement of BMP (Bone morphogenetic protein) signaling pathway (**Figure 4**) the BMP's led to the formation of heterotopic bone in soft tissues. shoulders, neck and spine are mostly affected (Kaliya-Perumal, Carney, and Ingham 2020). Flare ups and heterotopic

ossification usually manifest in the first decade of life in 95% cases. Minor trauma such as intramuscular immunizations, mandibular blocks for dental work, muscle fatigue, bruises, falls or influenza-like viral illnesses can trigger painful new flare-ups of FOP leading to progressive heterotopic ossification (Robert J Pignolo 2014).

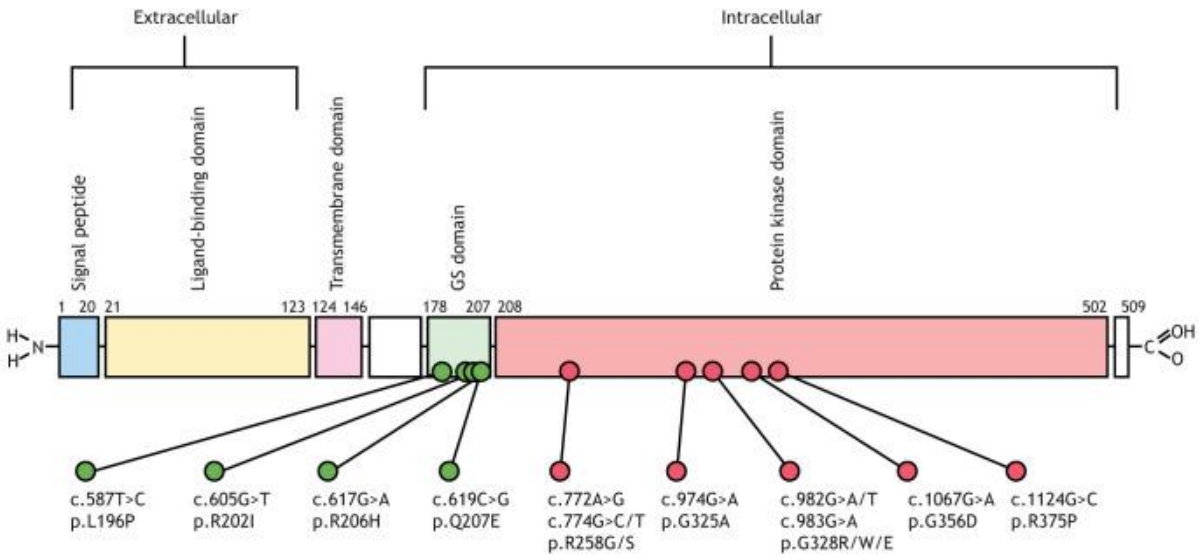


Figure 3. Schematic representation of human ACVR1, its various domains and locations of the mutations that have been causally linked to FOP. GS, glycine-serine. Adapted from (Kaliya-Perumal, Carney, and Ingham 2020) Abbreviation: GS-glycine-serine.

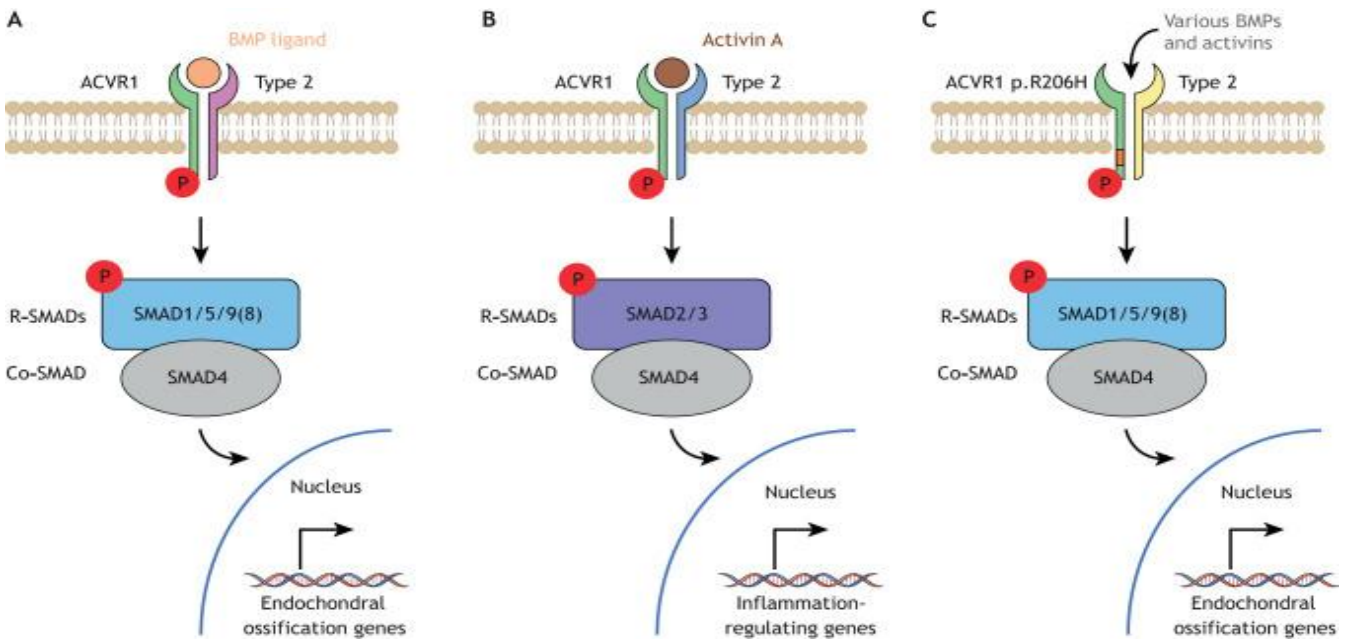


Figure 4.BMP signaling.
(A)BMPs bind to complexes of type I and type II serine/threonine kinase BMP receptors, such as ACVR1, on the cell surface to activate intracellular signal transduction via R-SMADs SMAD1/5/9(8). Phosphorylated SMAD1/5/9(8) forms a complex with co-mediator SMAD4 and translocates into the nucleus, where it regulates transcription that drives endochondral ossification. (B) On binding activin A, complexes of type I and type II BMP receptors activate intracellular signal transduction via SMAD2/3, which activates a transcription programme that regulates inflammation. (C) ACVR1 carrying a FOP mutation (most frequently the R206H substitution) in the intracellular glycine-serine domain not only yields enhanced response to various BMP ligands by initiating downstream signaling via SMAD1/5/9(8), but also responds to various activin ligands, thereby favoring endochondral ossification by triggering an osteogenic gene expression programme. BMP, bone morphogenetic protein; Co-SMAD, common partner SMAD; P, phosphorylation; R-SMAD, receptor-regulated SMAD. Adapted from (Kaliya-Perumal, Carney, and Ingham 2020).

Exosomal Vesicle Cargo Content

RNA Cargo: EVs are known to carry various types of RNA including non-coding RNA microRNA (miRNA), (**Figure 5**) messenger RNA (mRNA). mRNA is a single stranded molecule of RNA that corresponds to the genetic sequence of a gene. circular RNA (circRNA), long non-coding RNA's (Kyoung Mi Kim 2017). miRNAs are small, single stranded, non-coding RNA molecules containing ~22 nucleotides, which regulate gene expression predominantly at the post-transcriptional level. miRNA binds to the mRNA at the 3' untranslated region (UTR) of the targeted mRNA leading to the degradation of the mRNA or inhibition of its translation into proteins.

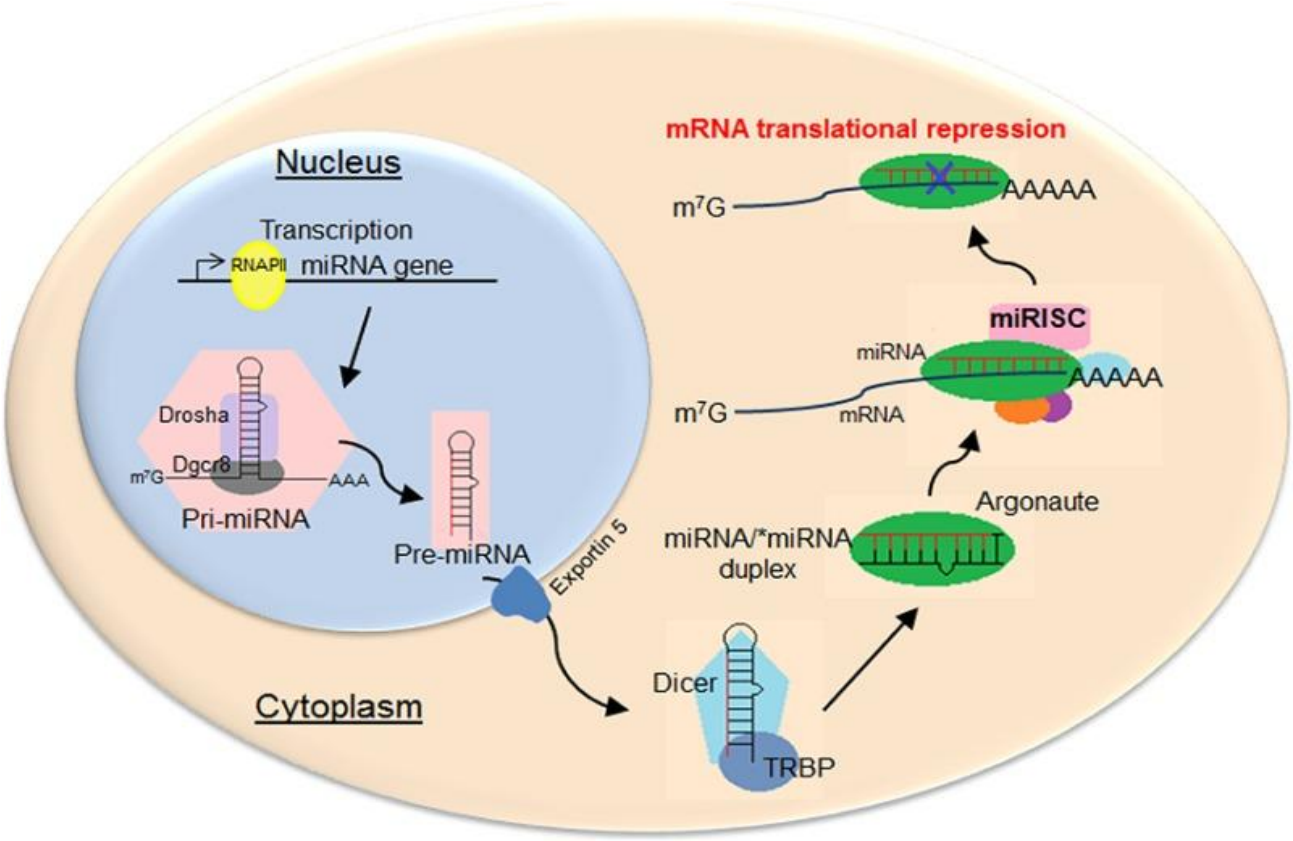


Figure 5. miRNA biogenesis and function in animals

A schematic of miRNA biogenesis and function in animals. miRNA biogenesis begins in the nucleus, where RNA-polymerase II-dependent (RNAPII) transcription of a relatively large capped and polyadenylated transcript known as primary miRNA (pri-miRNA). Pri-miRNA is processed by the RNase III endonuclease, Drosha, and its cofactor, Dgcr8 into smaller stem-looped structures known as precursor miRNAs (pre-miRNA). Pre-miRNAs are transported out of the nucleus by Exportin 5 into the cytosol, where further processing by a second RNase III enzyme, Dicer, leads to the generation of mature miRNA. The mature miRNA associates with the miRNA-induced silencing complex (miRISC), where Watson-Crick base-pairing between the seed-sequence of a mature miRNA and complementary sequences primarily located within 3'-UTRs of mRNAs results in post-transcriptional gene silencing.

Adapted from (Sachin Hajarnis 2015).

Victor Ambros and Gary Ruvkun were awarded the 2024 Nobel Prize in Physiology and Medicine for their groundbreaking discovery that revealed a completely new principle of gene regulation which is essential for all complex life forms (Danielle Gerhard 2024).

Proteins: Exosomes carry proteins including enzymes, receptors and signaling molecules, which modulate cellular functions in the recipient cells, proteins may act as messengers that influence various biological processes, to name a few immune responses, cell growth and apoptosis (programmed cell death).

Lipids: EVs are rich in lipids including cholesterol, sphingolipids and phospholipids which play a key role for membrane integrity, intracellular vesicle trafficking , recipient cell uptake and serve as signaling molecules (Jordan Fyfe 2023). Lipids play a crucial structural and regulatory role in extracellular vesicles influencing their interactions with target cells, biogenesis, cargo sorting and functional effects on recipient cells. They are essential for the structure and function of EVs. There are numerous diseases including cancer, metabolic and degenerative disorders and inflammation which are linked to the EVs lipid composition.

MATERIALS AND METHODS

Exosomal Vesicle Isolation from in vitro cultured dental cells.

Dental Cell Culture and Expansion:

This is an invitro research study. The study sample are dental cell lines that have been created from harvested, deidentified human teeth. The dental cell lines were expanded in vitro (**Figure 6**) then they were frozen down (cryopreserved). Thawed cryopreserved cells were then expanded in-vitro and used to generate dental cell EV samples, which were isolated from each cell line in triplicate. The following dental cell lines were analyzed: 1) three (3) independently isolated Wild type dental pulp stem cells (DPSC) lines; 2) three (3) independently isolated Wild type Periodontal Ligament Stem cell lines; and one (1) FOP-DPSC line (Fibrodysplasia Ossificans Progressiva).

For each cell line (3 WT DPSCs, 3 PDLCs, 1 FOP DPSC Line), cryopreserved cells were selected from Cryomap (Yelick Lab log of all cryopreserved cells), cryovial was removed from the liquid nitrogen tank and immediately place in the tissue culture hood at room temperature (RT). Media was warmed up in water bath (37^0 C), cells were thawed until slushy, 9ml of prewarmed media was added pipetting up and down till everything was mixed in the tube. The total volume of media was 10ml, it was then centrifuged at 1,100 RPM for 5 minutes at room temperature (RT) by adding balance, supernatant was gently poured off avoiding disruption of the pellet, the bottom of the tube was gently rubbed along the grate to dislodge the pellet, the pellet was then resuspended in 10ml of pre-warmed fresh media. It was then seeded into a T75 tissue culture flask and finally

placed in the incubator. The cells were grown until they were 80-90% confluent (**Figure 7**) at 37-degree Celsius, 5% CO2.

To obtain enough cells for EV isolation, we next expanded the cells in vitro. Briefly, Wild Type (WT) Dental Pulp stem cells (DPSC), Wild Type (WT) Periodontal Ligament (PDL) cells and FOP-DPSC stem cells were seeded into three different T75 flasks (seeding density ≈2.5 x 10^6 cells). Briefly, each T75 containing confluent cells were passaged to T175 flask and the confluent T175 was expanded to 4T175 flasks, Three T175 were cultured for exosome isolation and the remaining T175 flask was used for the next round of cell expansion.

a. T75 Flask: 175 cm2 area

b. 2.5 x 10^6/145 *175=3 x 10^6

Expanded cells to: 9 x 10^6 then seeded 3 x 10^6 into 3 T75 flasks, changed media every other day until the cells are 80% confluent.

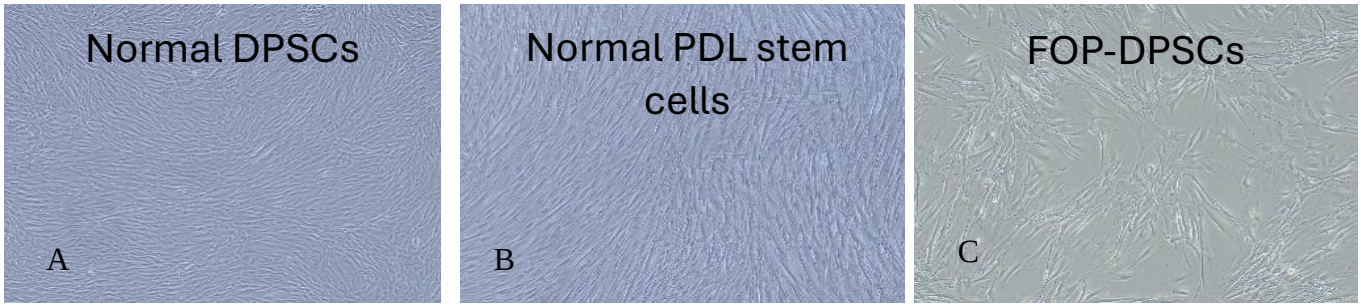


Figure 6. Title: Dental Cell Culture – Confluency of cells.

Isolation of EVs from Dental Cells cultured in Normal Media (NM) and Osteogenic media (OM)

We needed ~ 6 x T 175 flasks of cells for exosome isolation. We also needed one additional T175 flask to passage for the next round of Exosome (EV) isolation. We passaged the confluent T175 flask cells (~20.0 x 10^6 cells) to new 3xT175 flasks. When confluent, expand the 3X T 175 flask to 7 X T 175 flasks for the first two expansions and one (1) T175 flask for next round of cell expansion. From the 6X T175 flasks, 3xT175 flasks cells were grown to at least 90% confluency in growth media and 3xT175 flasks in osteogenic media. For 3 of the confluent T 175 flasks, switched media from serum containing media to serum free growth media. Serum containing media was removed, cells were washed twice (2X) with sterile PBS and media was replaced with serum free media.

For the other 3 of the confluent T 175 flasks, we switched media from serum containing osteogenic media to osteogenic serum free media. Serum containing osteogenic media was removed, cells were washed twice (2X) with sterile PBS and media was then replaced with osteogenic serum free media. Cells were grown for 72 hours in serum-free media or osteogenic serum free media, respectively.

For the 7th T 175 flask, was maintained in serum containing media, and passaged to a new (3) T 175 flask for the next round of EV isolation.

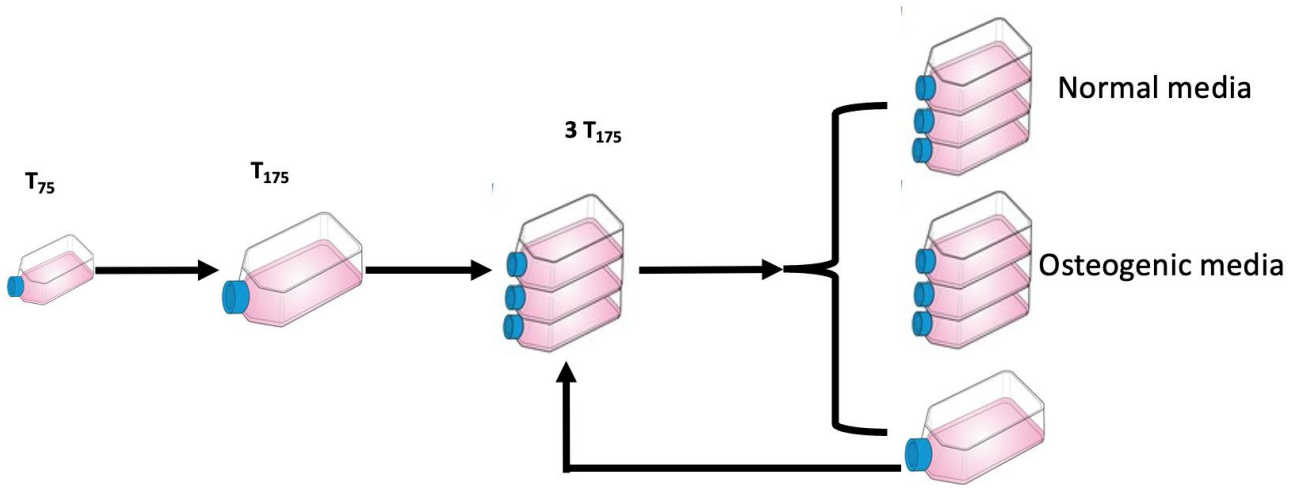


Figure 7. Dental cell expansion

Osteogenic media (OM): Similar cell expansion was performed for cells cultured in osteogenic media. OM is formulated to promote the differentiation of mesenchymal stem cells into bone forming cells (osteoblasts). The media consists of Advanced DMEM,10% fetal bovine serum (FBS),1X Glutamax,100nM Dexamethasone,10nMbeta glycerol phosphate,50ug/mL Ascorbic acid,1X PSA (penicillin, streptomycin and amphotericin).

Serum Free Media Culture

For each cell type and media type, three T175 flasks in normal media were switched to serum free media and osteogenic serum free media respectively to isolate EVs from cell culture media, Serum was removed since serum contains EVs, and we did not want to contaminate our Dental Cell EVs with serum EVs. After the cells were switched to serum free media, cells were cultured invitro for 72 hours, at which time we then collected the media containing secreted EVs. EVs were either isolated immediately after collection, or from collected media that had been stored

and frozen at -80^{0}C, for immediate EV isolation we spun the media for 3,500g in centrifuge for 5mins and continued to exosome isolation. For later EV isolation, EV containing media was spun for 3,500g in centrifuge for 5mins and stored at - 80^{0}C. Right before exosome isolation, the frozen media was thawed in 37^{0}C water bath (~20 min).

Exosome Isolation (using size exclusion chromatography).

Collect Cell culture media	**Centricon Plus-70 *30K*** Max load = 70mL Min load = 10mL	**iZON qEV 35nm Gen2 gel filtration** Max load = 500uL Final collection = 1.5mL	**Amicon Ultra-4 *30k*** Max load = 4mL Final concentrate = ~50uL

Figure 8. Columns used for Exosome isolation.

For exosome isolation, the following protocol was used. EVs were harvested by successive filtration using Centricon filters (Millipore, Burlington, MA), Izon columns (Izon sciences, New Zealand) and Amicon filters (Millipore, Burlington, MA) (**Figure 8**). The three primary categories of size -based methods for exosome isolation are ultrafiltration, sequential filtration and size-exclusion chromatography (Dilsiz 2024).

Centricon Plus-70 30K: Media was thawed and centrifuged at 2,000g for 10min at 4^0C to remove any cell debris. Supernatant containing EVs was saved, and the pellet was discarded. Media was poured in centricon filter and centrifuged at 3,500g for 30mins at 4^0C, centricon filter was then turned upside down and placed in the collection cone and centrifuged at 1,000g for 2mins at 4^0C was now filtered through 0.22um filter into a new Eppendorf tube. The tube was kept on ice and the next filtration step to Izon column was continued.

iZON qEV35nm (gen 2): Izon columns use size exclusion chromatography that is efficient for extracellular vesicle isolation, larger entities precipitate out of the gel first, followed by smaller free proteins-tapering off in concentration. Once the column is no longer dripping after wash, place the 3ml collection tube (Eppendorf tube) underneath the column. Add max 500uL media concentrate collected from the Centricon to the Izon column, wait until fully absorbed by gel column (no dripping). Add PBS to the buffer reservoir, discard the first 3mL as they are the fractions containing larger particles from media. As the optimal fraction size is 400uL, those 2mL represent the first 5 fractions. The 2mL tube was removed and replaced by another Eppendorf tube, 6-10 fraction (2mL total) was collected in an eppendorf tube, this pool contains exosomes.

Fractions 11+ contain soluble proteins. The exosome content was checked and analyzed using Zetaview, Nanoparticle tracking instrument (NTA).

Size exclusion chromatography (SEC) also known as gel filtration was first invented in 1955 by Grant Henry Lathe and Colin R Ruthven, who used swollen starch granules to separate biopolymers based on size (Dongbin Yang 2020).

Amicon Ultra-4 (30k): Used for final concentration of the exosome sample. Max 4mL of exosome sample was added to the Amicon column of the above collected volume from Izon column and centrifuged at 3,500g for 30 mins at 4^0C. The final concentration obtained ranged from 50uL-80uL, the tubes were labelled and stored at -80^0C.

Exosomal Vesicle Characterization.

EV Yield and Size. EV yields and sizes were characterized by nanoparticle tracking analysis (Zeta view Particle Metrix, Ammersee, Germany) (**Figure 9**) 1mL of the sample was loaded into the zeta view instrument. Zetaview is a unique nanoparticle tracking analysis instrument for measuring hydrodynamic particle size, zeta potential, concentration and fluorescence.

Total RNA isolation from in vitro cultured Dental Cells.

Rationale: Dental cell harvest is done to potentially preserve and leverage the cells for future regenerative therapies; they have the potential to differentiate into various cell types. They show promise in regenerative dentistry and other regenerative therapies. In this study it is done to compare the mRNA libraries constructed from EVs and the cells that were harvested after collecting the media for exosome isolation for future studies. RNA isolation is done to extract high quality RNA from cells which is crucial for accurate analysis of gene expression and experiments that include RNA sequencing and cDNA library construction.

All procedures were conducted in the Tissue Culture Hood (Biosafety Cabinet, room M815A). Collect serum free media from all flasks for exosome isolation, wash cells with (2X) PBS, add 20mL per confluent T175 flask and 10mL per confluent T75 flask. Gently using 10mL pipette resuspend cells in Trizol, avoiding bubbles, when cells were suspended and transferred to Trizol-cell suspension to an RNAse free tube, tubes were frozen with cells in -80-degree Celsius freezer.

EV RNA Cargo

Next, once dental cell EVs were isolated, we next wanted to determine what RNAs were inside EVs isolated from each cell type under each tissue culture condition. Differential small RNA profiling was used to define miRNA profiles of each dental cell type. RNA was extracted from exosome using QIAGEN QIA Symphony SP/AS with QIA Symphony RNA Kit and miRNA CT 400 protocol. Extracted RNA was concentrated via SpeedVac and quantified using 5200 Agilent Fragment Analyzer version 3.1.0.12 (Agilent Technologies, Santa Clara, CA).

Isolated EV RNA was used to create sequencing libraries using QIAseq miRNA library kit (Qiagen, Germany): following manufacturer protocol (Tufts University Genome Center Core Facility Tufts Medical School, Boston MA). Molar concentration of resulting libraries was determined on Agilent Fragment Analyzer. The sequencing was performed on an Illumina NovaSeq X Plus using 1.5B 100 cycles chemistry. Base calling and demultiplexing on raw sequencing data were performed with Illumina Bclconvert, resulting in compressed fastq files. Fastq files were processed with Cutadapt to remove the internal adaptor sequence and separate target reads and unique molecular identifier (UMI). The Cutadapt outputs were then processed with a customized Perl script to filter poor quality reads and collapse UMI into a UMI removed fastq file.

Reads contained within the resulting files are then mapped to the human genome (UCSC hg38) using HISAT v2.0 and created BAM files as output. The BAM files were used as input for read counting using feature Counts (Subread package) and Gene code (v20) gene annotation file. The resulting count table was used as input into Qlucore Omics Explorer (v3.10) for differential expression analysis and visualization. The p-value for pairwise comparison was done with Mann-Whitney and the p-value cutoff was <0.05.

Exosome protein extraction and quantification.

Protein extraction is the first step required for Western Blot, Western Blot is a technique used to identify specific protein markers present in isolated exosomes, thus validating successful EV isolation. EV protein quantification is conducted to make sure that we use similar amounts of protein for all Western blot analysis.

For protein extraction EVs were lysed with RIPA buffer (Thermo fisher, USA) and protease inhibitor cocktail (Sigma- Aldrich, St Louis, USA). 1 µL of protease inhibitor cocktail to 20µL of RIPA buffer to an Eppendorf tube. 79µl of the exosome sample was aliquoted to the same Eppendorf tube and mixed well, sample was incubated on ice for 20 mins with periodic mixing for ~ every 5 minutes.

Next, BCA assay was conducted to determine the total protein concentration of extracted EV proteins. BCA assay kit was used (Abcam, USA) as per manufacturer's suggested protocol. A 200µg/mL BSA standard working solution was prepared by diluting 0.5mL of BSA standard in 4.5 ml of de-ionized water or the diluent (1x RIPA buffer). Using this working solution.

Table 1. BSA standard solutions table.

Tube #	Volume of BSA (mL)	Volume of Diluent (mL)	Final BSA Concentration (µg/mL)
1	1 mL of 200 µg/mL Stock	4	40
2	4 mL of tube 1	4	20
3	4 mL of tube 2	4	10
4	4 mL of tube 3	4	5
5	4 mL of tube 4	4	2.5
6	3.2 mL of tube 5	4.8	1
7	4 mL of tube 6	4	0.5
8 (Blank)	0	8	0

Next, BSA Standard solutions was prepared as indicated as seen in Table 2. EV protein samples were diluted using de-ionized water with concentrations within the assay range (0.5-40 µg/mL).

For exosomes, prepare a 1:10 and 1:20 Dilution:

1:10 Dilution, 15uL of sample to 135uL of 1x RIPA buffer

1:20 Dilution, 7.5uL of sample to 142.5uL of 1x RIPA buffer

150 µL of each BSA Standard and protein samples were added into separate Eppendorf tubes. 150uL of BCA reagent working solution was added to each well containing BSA standards and samples were centrifuged for 2 secs, the plate was sealed with an adhesive plate sealer and incubate at 37^0C for 2 hours. After incubation, the plate was cooled to room temperature to ensure that there is no liquid on the plate sealer. The absorption wavelength of the microplate reader was set to 562nm, and the absorbance was recorded (OD562) of all BSA standards and samples. The plate layout, sample absorption, concentration and standard curve was exported to a spreadsheet **(Figure 12**).

Western Blot Analysis

Western blot analysis was conducted on EV protein extracts (~5µg) to define and study the protein content in each cell type and cell protein extract (Cell lysates) was used as a positive control (15µg).

Table 2. Protein sample preparation.

Cell type	Sample	Lysis buffer	Laemmeli dye	Total
FOP-DPSC	13µg	2µg	5µg	20µg
WT-DPSC	9µg	3µg	4µg	16µg
PDL	11µg	4µg	5µg	20µg
FOP-BMP	3µg	6µg	3µg	12µg

Western blot protein samples were prepared as seen in Table 3, Briefly.

Protein samples were prepared with an appropriate amount of 4x sample loading buffer (4x Laemmli Sample Buffer, 1610747 Bio-Rad Hercules, CA, USA) for 4X laemmli Dye add 10% Bone morphogenetic protein (BME) (i.e. 900uL of laemmli dye plus 100uL BME). Denature samples by boiling for 5 minutes and then immediately place on ice. Spin samples in centrifuge for 30 sec (Eppendorf centrifuge 5417R, Germany).

Gel electrophoresis using 4-15% Gradient Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Isolated EV proteins were then size fractionated using SDS-PAGE as follows. Briefly, the running buffer was prepared. Prepare running buffer (100mL of 10x Tris-Glycine Buffer + 900mL of diH2O). Remove the gel from its packaging and rinse it off with diH_2O. Make certain to remove the gel comb and the tape covering the bottom edge of the gel. Assemble the gel apparatus with the wells of the gel to the inside of the chamber. The inside of the inner apparatus chamber was filled with a running buffer so that the wells of the gel are covered and then fill the rest of the apparatus with the remaining running buffer. A pipette was used to remove any remaining bubbles from inside the wells. The amount of protein marker ladder for iBright used was 3uL, and for SeeBlue we used 10µL. Each sample was loaded into its respective well. Load 10-11 µl of the standard protein ladder. Lid was placed on the apparatus and attached to the power source. Run the gel at a constant of 130 V for 60 minutes or longer until the standard protein ladder has migrated to satisfaction. The gel apparatus was turned off and the gel was removed and transferred to a PVDF membrane immediately.

Next, size fractionated proteins were transferred from the Gel to a Polyvinylidene fluoride (PVDF) membrane (BIO-RAD, USA). Briefly, transfer buffer 10X (Bio-Rad, USA) was prepared from Bio-Rad 10x Tris/Glycine/SDS Buffer (Cat #1610732, mix 100 ml of Tris/Glycine10X buffer with 900 ml diH_2O). Transfer buffer needs to be fresh and cold. Membrane was then cut to an appropriate size, labeled and soaked in methanol for 5 minutes then in diH2O for 5 minutes followed by cold transfer buffer for 5 minutes prior to use. Using a cassette opener, the gel cartridge was opened, and the outer plastic was discarded, the gel was removed carefully, and the top portion was cut to include the region that has the protein of interest. It was then soaked in pre-chilled transfer for 10 min and kept chilled. The transfer sandwich was set up and placed into the transfer apparatus according to color, the membrane had to be submerged in transfer buffer, and run at 70V for 1 hour, after the timer went off carefully the cartridge was opened, the blotting paper and the gel was discarded.

After the transfer, the PVDF membrane was checked (1620174. Bio Rad, USA) for the pre-stained standard, which indicated that the transfer was successful. The membrane was blocked with 5% Bovine Serum Albumin (BSA Fraction V, Sigma Aldrich, Mannheim, Germany) (50mL TBST,2.5g BSA) and Tris buffered saline Tween-20 (TBST Thermo scientific, Rockford, IL, USA) (950mLdiH2O,45mL,1X TBST). Once BSA was fully dissolved we ensured that the final volume was 50mL, we then submerged the membrane (about 15mL of 5% BSA) and put on a shaker at room temperature (RT) for 60 minutes. After blocking we washed with TBST 3X, 10 minutes each. The membrane was cut at this stage to probe for different proteins. A dilution of the primary antibody in TBST was prepared. The primary antibodies used for Western blotting were Alpha-actin control (A169, Millipore, A2066, Rabbit 42Kda, dilution 1:2000), CD-81 (A 180,

Santa Cruz, SC-13118 mouse,22-26kDa dilution 1:500, Alix dilution 1:250). The PVDF membrane was incubated in the primary antibody solution overnight at 4°C on the rocker. The PVDF membrane was washed 3X in TBST, each wash with a duration of at least 10 minutes. Appropriate dilution of secondary antibody (1:1000) Anti-Rabbit IgG and Anti-Mouse IgG was prepared, and the membrane was incubated for 1 hour at room temperature. Dilutions used: Alix-1:250, CD81-1:500, Actin-1:2000.

The PVDF membrane was washed 3X in TBST, each wash with a duration of at least 10 minutes. The membrane was stored in TBST until used. Do not allow the membrane to dry out. Western blot imaging and analysis were done with Imager LICOR Odyssey CLX (Nebraska, USA) (**Figure 11**).

STATISTICAL ANALYSIS

Statistical analysis included Welch's ANOVA, Kruskall- Wallis Test, Independent samples t-test and Mann-Whitney U Test using SPSS software, version 29.0.2.0(20) IBM SPSS Statistics. $p<0.05$ was considered to indicate a statistically significant difference. No statistical differences were observed in dental cell EV size/yields.

RESULTS

In this study, EV yield and size were compared in 3 different dental cell types (Wild type DPSCs, Wild type PDL, FOP cultured in normal media (NM) and osteogenic media (OM).

Dental cell secreted EV isolations and characterizations

EV yield was uniform with average yields 8.67E+09 (± 2.15E+09) particles and EV size range was ~104.16nm ± 3.53nm (Figure 9).

Zetaview reports as seen after every EV isolation.

Zetaview Images.

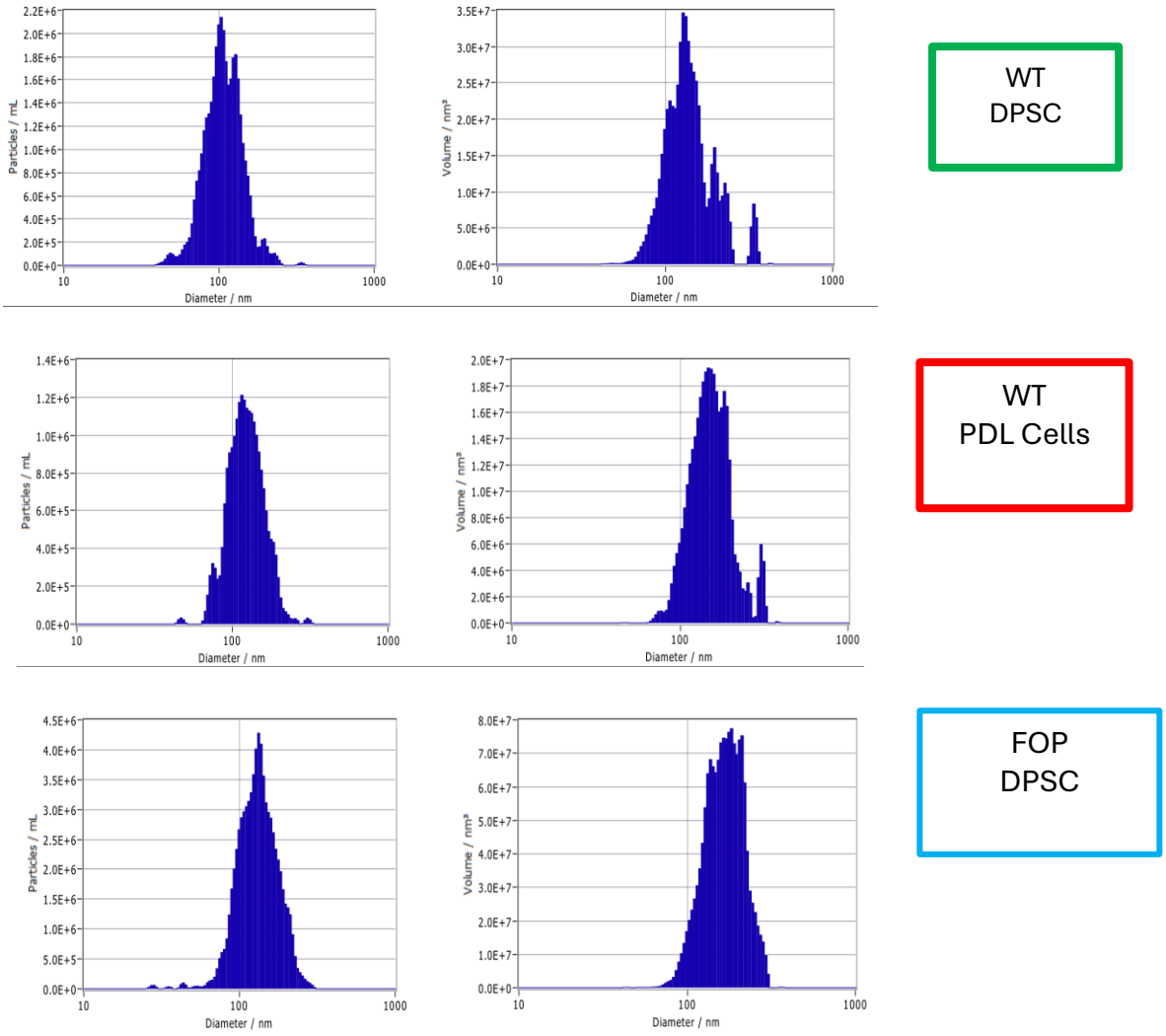


Figure 9. EV Particle yield and size distribution. Blue Box: FOP patient-derived DPSCs. **Red Box:** WT PDL Cells. **Green Box:** Wild-type DPSCs. EV yield and size range were relatively uniform with an average yield of 8.67E+09 ± 2.15E+09 and a diameter of ~104.16nm ± 3.53nm.

Using our method we got reliable EV results (Table 4). EV yields and EV RNA yields are seen in 3 different dental cell types and their replicates in both normal and osteogenic media.

Table 3. Dental cell secreted EV isolation and characterizations.

Cell type	*Media*	*Replicate*	*EV Yields#*	*EV RNA Yields*
			Particles	***ng/uL***
DPSC	*NM*	*1*	*2.40E+10*	*0.5151*
		2	*3.60E+09*	*0.2306*
		3	*4.00E+09*	*N/A*
	OM	*1*	*9.00E+09*	*0.0387*
		2	*4.20E+09*	*0.0447*
		3	*6.40E+08*	*0.155*
FOP-DPSC	*NM*	*1*	*4.00E+09*	*0.1757*
		2	*3.60E+09*	*0.1251*
		3	*1.02E+09*	*0.1733*
	OM	*1*	*3.20E+09*	*0.2528*
		2	*6.40E+09*	*0.1235*
		3	*3.60E+09*	*0.2481*
PDL	*NM*	*1*	*7.40E+09*	*0.0357*
		2	*4.05E+09*	*0.0361*
		3	*3.00E+09*	*0.184*

EV size ranges varied, but when normalized were not statistically significant p> 0.05 (**Figure 11**).

Small RNA was isolated from in vitro cultured dental cell EVs.

Small RNAs are a class of regulatory molecules, including miRNAs, siRNAs, and piRNAs, that play crucial roles in gene expression and various cellular processes.(Howida M.Nail 2023). Small RNA libraries were created to enable the sequencing and analysis of these small RNAs. Once

identified, small RNAs will be used to characterize novel small RNAs for each dental cell type to determine the expression levels of identified small RNAs and to study their relationship to human health and diseases. Small RNA libraries were successfully created by the TUSDM Genome Center (Boston, MA), Libraries were sequenced on a 5200 Fragment Analyzer, version 3.1.0.12. Small RNA characterizations were then performed to identify small RNAs, including microRNAs (miRNAs) present in each type of isolated EV miRNA data analysis in all 3 cell types (Table 5). We next conducted comparisons of EV isolated micro-RNAs.

Table 4. EV micro-RNA library comparison

DPSCs	***FOP- DPSCs***	***PDL Cells***
Normal Media	*Normal Media*	*Normal Media*
Osteogenic Media	*Osteogenic Media*	

Results of EV protein extraction and quantification.

EV proteins were isolated using RIPA buffer+ Protease inhibitor cocktail EV protein concentrations were next determined using BCA Assay as per Manufacturer's Specifications (Abcam, USA) EV protein Concentrations are shown in Table 1.

Table 5. Dental cell EV Protein Concentration.

	Protein concentration	**Total protein**
FOP	0.39672ug/µL	31.249µg
DPSC	0.583056ug/µL	17.701µg
PDL	0.466389ug/µL	14.159µg

We used standard curve **(Figure 10**) to measure concentration of protein yields. The curve is a representation of BCA readouts.

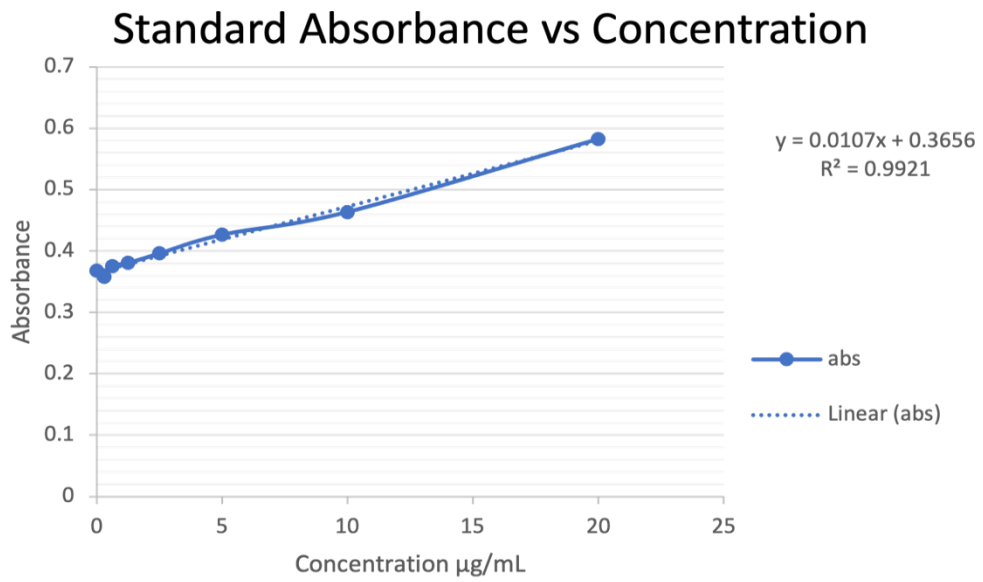
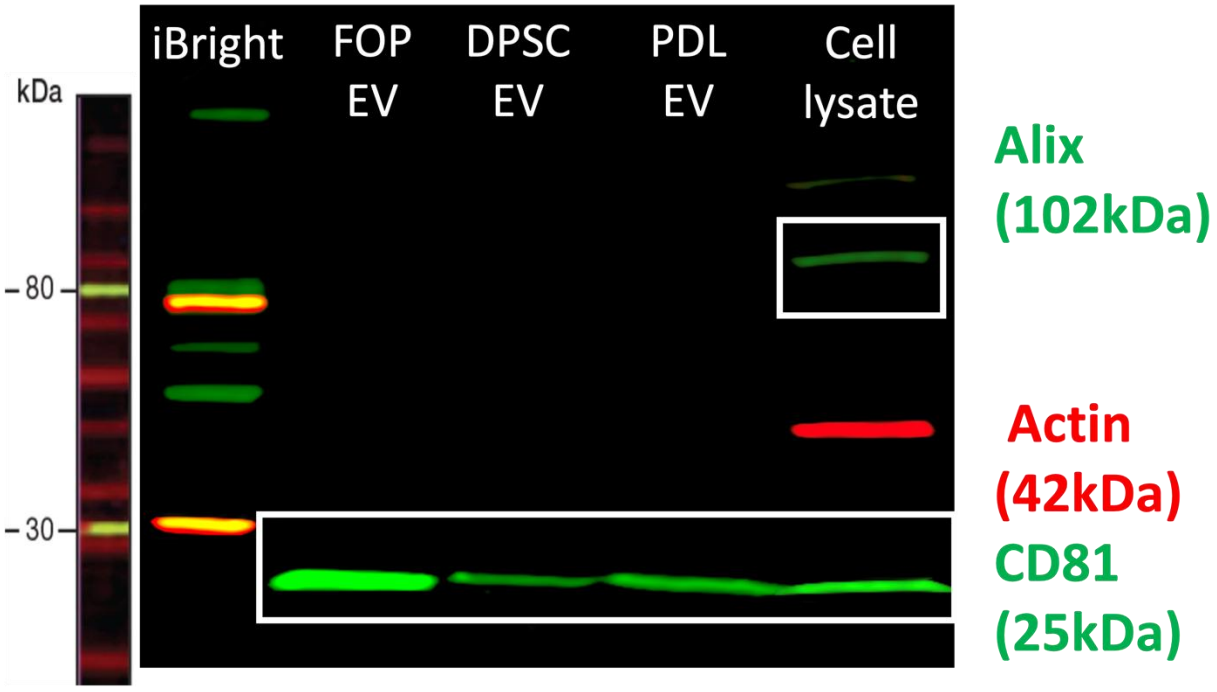


Figure 10. Standard curve of BCA assay analysis done for total protein concentration.

Figure 11. Western Blot Analysis. EV marker CD81 is expressed in all three types of dental cell EVs.



Western blot was performed to study and define protein content on EV protein extracts, (~5 µg), cell protein extract (15 µg) was used as a positive control .CD81 expression validated EV protein isolation. Actin was not detected in EV protein extracts. Alix is also not detected. We have worked on several WB to prove several extractions in different cell types and isolation methods. In conclusion our dental cell EV protein does not cross react with these antibodies.

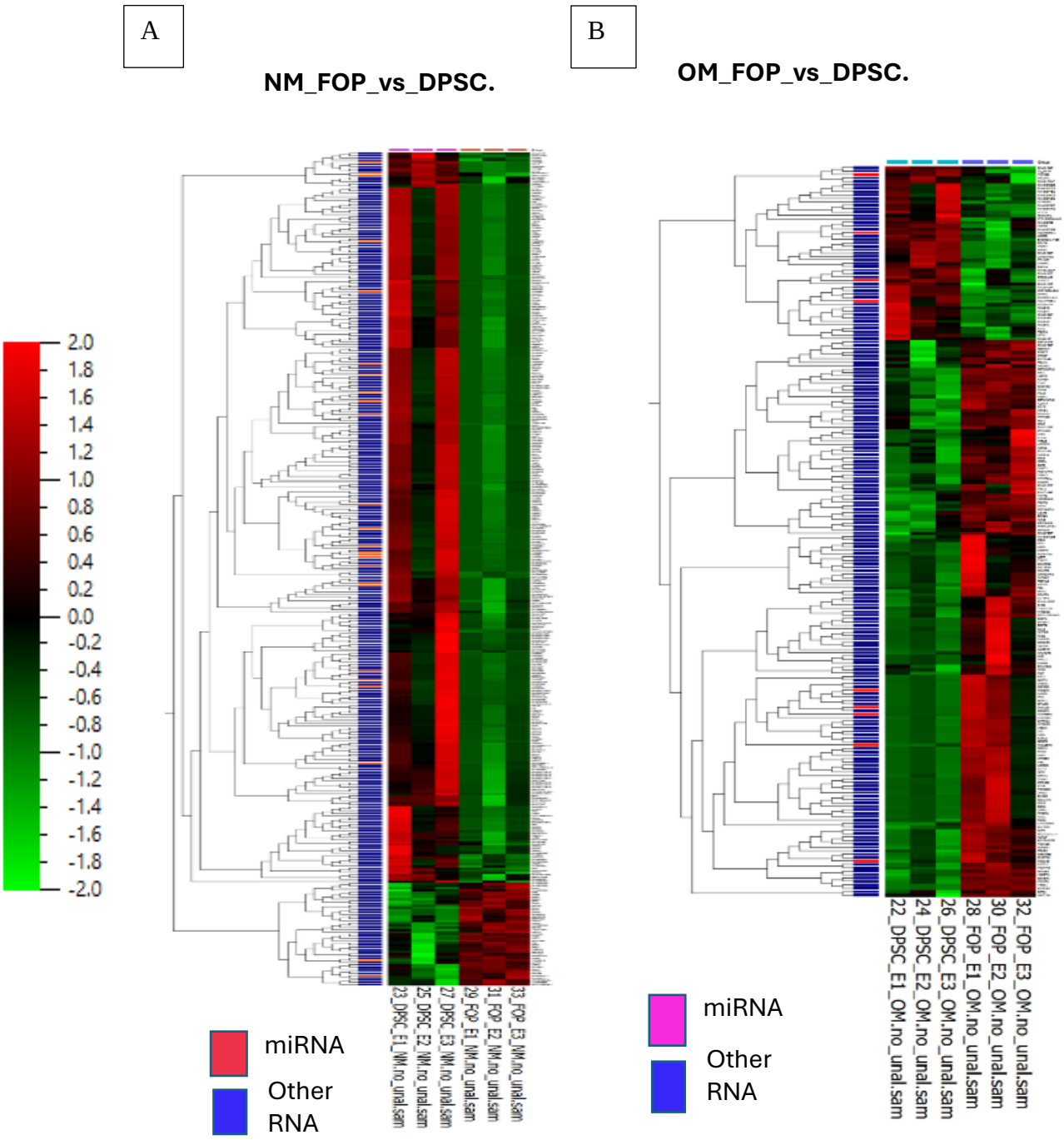


Figure 12. EV miRNA profiling and Heatmap comparisons. NM FOP vs DPSC and OM FOP vs DPSC. (preliminary results).

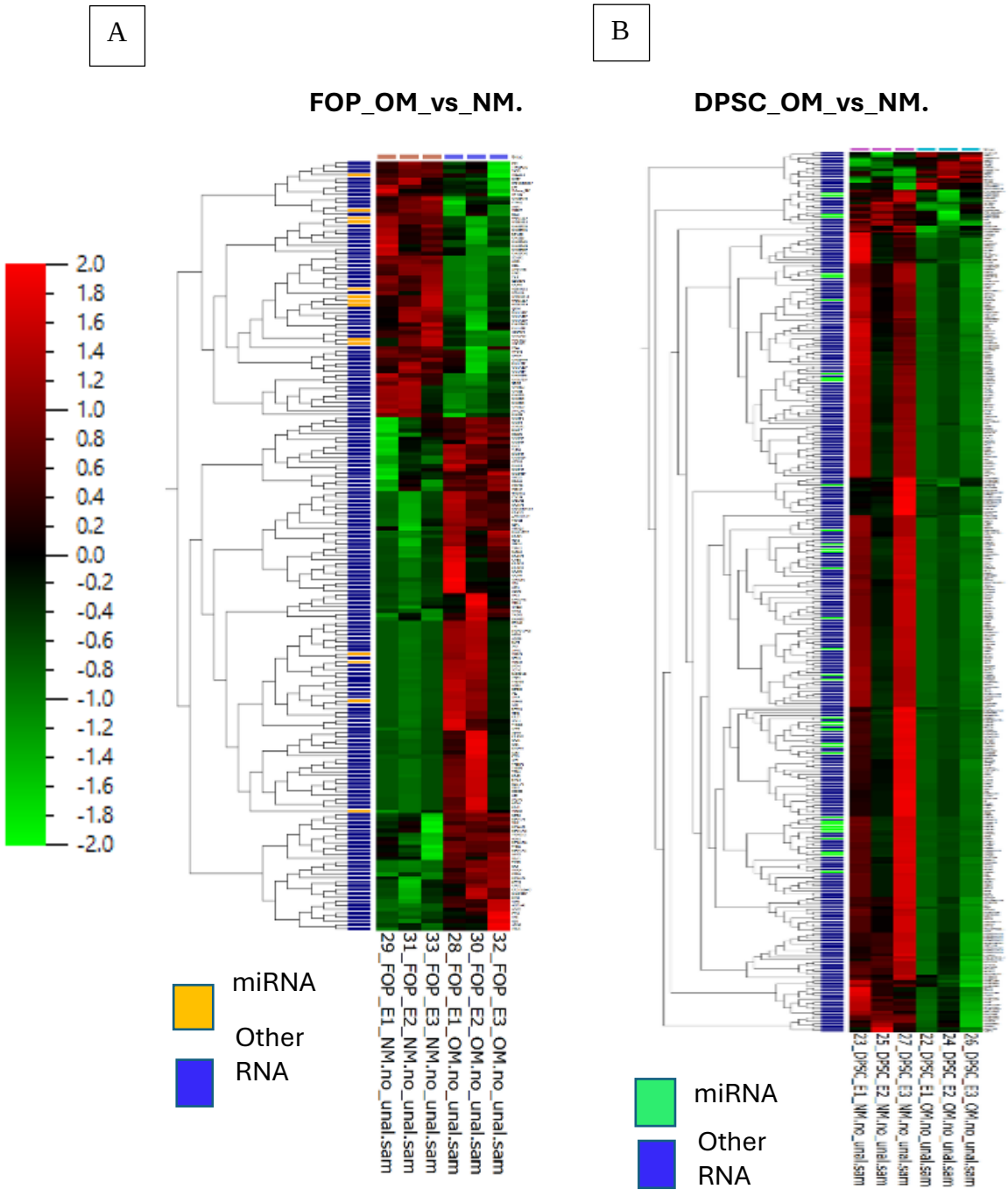


Figure 13. EV miRNA profiling and Heatmap comparisons. FOP OM vs NM and DPSC OM vs NM (preliminary results).

Table 6. Detailed EV miRNA heat map comparisons.

FOP NM vs DPSC NM			
Comparison		**miRNA**	**Fold change**
Increased	1	MIR486-2	23.66
	2	MIR335	16.65
	3	N/A	N/A
Decreased	1	MIR483	0.03
	2	MIR483	0.04
	3	MIR210	0.05

FOP OM vs FOP NM			
Comparison		**miRNA**	**Fold change**
Increased	1	MIR324	9.35
	2	MIR379	7.97
	3	MIR483	6.4
Decreased	1	MIR877	0.04
	2	MIR486-2	0.08
	3	N/A	N/A

DPSC OM vs DPSC NM			
Comparison		**miRNA**	**Fold change**
Increased	1	N/A	N/A
	2	N/A	N/A
	3	N/A	N/A
Decreased	1	MIR158	0.05
	2	MIR185	0.07
	3	MIR361	0.07

FOP OM vs DPSC OM			
Comparison		**miRNA**	**Fold change**
Increased	1	MIR486-2	19.08
	2	MIR139	9.84
	3	MIR27A	8.12
Decreased	1	MIR877	0.09
	2	MIR483	0.46
	3	N/A	N/A

Upregulation and downregulation of top 3 miRNA expression levels seen in FOP-DPSC and WT-DPSC in normal and osteogenic media.

DISCUSSION

Dental cell EV characterizations and comparisons.

The objective of this study is to characterize dental cell secreted Exosomal Vesicles (EVs) for future applications in Craniomaxillofacial (CMF) tissue regeneration. Our approach was to isolate and compare the secreted EVs and their miRNA cargo from the conditioned media of three types of in vitro cultured dental stem cells-1) Wild type dental pulp stem cells (DPSC), 2) Wild type periodontal ligament stem cells (PDLSCs) and 3) Fibrodysplasia Ossificans Progressiva stem cells (FOP-DPSCs). The emerging role of exosomes as mediators of intercellular communication and their potential involvement in human health and disease has led to an extensive and rapid development of methods used for exosome isolation techniques and characterization (Agata Abramowicz 2018). In normal and pathophysiological conditions, EVs play a pivotal role delivering biological messages between cells. (Menghong Wang 2020). Due to their characteristics and functions, they can be utilized as novel biological platforms for both diagnosis of diseases and therapeutics.

This study has successfully isolated EVs from 3 different cell types, Wild type DPSC, Wild type PDL and FOP-DPSC respectively, that had been cultured in vitro in normal media or in osteogenic media. We then successfully characterized the size and yield of exosomes derived from in vitro cultured WT DPSC, WT PDL stem cells and FOP-DPSCs by using nanoparticle tracking analysis. Our findings align with previously published reports that used size exclusion chromatography method to isolate EVs which preserves the integrity of EVs, as well as EV

structure and biological activity (Laura M Doyle 2019). The iZON column allows for rapid, cost-effective high precision exosome isolation. Altogether, a combination of techniques is required to isolate and characterize EVs including size and quantification.(Dilsiz 2024).For example, Nanoparticle tracking analysis (NTA) method not used in this study, allows for the detection of EV morphological structure, concentration and size, it can also detect extracellular vesicles of all shapes and sizes with diameters as small as 30nm as mentioned in previous studies (Dilsiz 2024). In this study, we found that dental cell harvested EV particle size distribution was relatively uniform with an average diameter of ~104.16±3.53nm in all 3 dental cell types.

miRNA cargos of dental cell exosomes.

As previously described, EVs contain a vast range of molecules including DNA, proteins, lipids, RNA, long noncoding RNAs and small RNAs including miRNA, circular RNA (circRNA) and piwi interacting RNAs (piRNAs) (Howida M.Nail 2023).Micro-RNAs or miRNAs are highly bioactive molecules that have attracted much interest because their ability to regulate post - transcriptional gene expression through degradation of mRNA transcripts and through inhibition of translation(Pratibha potla 2021). miRNAs are also the most numerous exosomal cargo molecules (Dongdong Zheng 2021). In this study, we were particularly interested in the small RNA cargo present in each type of isolated dental cell EVs. Small RNA sequencing libraries were constructed from isolated dental cell EV RNA using QIASeq miRNA library kit (Qiagen, Germany). Next, Differential small RNA profiling was used to define miRNA profiles of each dental cells secreted EV type. Analysis of the resulting miRNA profiles provided the following insight into each type of dental cell EV cargo. In the detailed EV miRNA heat map comparison performed on

FOP-DPSC and Wild type DPSC cultured in normal tissue culture media (NM), we found a decrease in miRNA expression with MIR486-2, MIR335 and increased expression of MIR483 and MIR210.

In comparing EV miRNA harvested from WT DPSC cultured in OM vs those present in EVs harvested from WT DPSC NM there is an increase in miRNA expression with MIR158, MIR185, MIR361. In FOP-DPSC OM vs FOP-DPSC NM there is a decrease in miRNA expression seen in MIR324, MIR379, MIR483 and an increase in miRNA expression with MIR877, MIR486-2. In FOP-DPSC OM vs WT DPSC OM there is a decrease in miRNA expression seen with MIR486-2, MIR139, MIR27A and an increase in miRNA expression with MIR877, MIR483, respectively. For EV protein profiling Western blot analysis was conducted to validate EV isolation.

Methodological considerations.

We would like to mention a few considerations when assessing our study results. While our use of nanoparticle tracking analysis (Zetaview) provided reliable EV particle size distribution, variations in size and yield were observed across the collections, even among the samples originating from the same dental cell type. This can be explained by the growth patterns seen in our 3 types of dental cell lines which were influenced by several factors like cell type, culture conditions such as nutrient availability, temperature, pH and environmental stress like nutrient levels and presence of toxins. The degree of confluency of cells may contribute to the variability observed across different cell types. Furthermore, RNA Libraries were sequenced on a 5200 Fragment Analyzer, version 3.1.0.12 which gave valuable insights into the RNA profiles which aims to determine the expression levels of different miRNAs.

Future Perspectives.

Ongoing studies include analysis of miRNA profiling to identify key miRNAs regulating dental cell differentiation in health and disease. Mechanistic analyses of identified miRNA in health and disease will be validated in subsequent in- vitro studies. We anticipate that functional characterization of EV cargo in each dental cell type will help define roles for dental cell EV signaling in oral health and disease.

CONCLUSIONS.

We have successfully isolated EVs from in vitro cultured dental cells. Comparable EV yields were obtained from all dental cell lines. EV particle size distribution was relatively uniform with an average diameter of approximately ~104.16nm ± 3.53nm. No statistical differences were observed in dental cell EV size/yields in all 3 cell types. Western Blot analyses validated EV protein isolations. miRNA isolation and profiling were successful for all dental cell lines. Ongoing analysis of dental cell secreted EV miRNA comparisons are expected to identify novel miRNA mediated signaling pathways for each type of dental cell EV.

In conclusion, we anticipate that our ongoing and future studies will reveal the promise of dental cell exosomes in diagnosing and treating a variety of Dental, Oral and Craniofacial diseases and conditions. We look forward to pursuing strategies to develop novel EV based therapies for functional repair of craniomaxillofacial defects. We anticipate that dental cell secreted exosome research will continue to advance, leading to innovations in the diagnosis and treatment of Dental Oral and Craniofacial (DOC) diseases.

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