Comprehensive Proteomic Analysis of Mesenchymal Stem Cells- and Osteoblasts-derived Extracellular Vesicles of Osteogenesis Imperfecta Patients

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Abstract

Introduction: Osteogenesis Imperfecta (OI) is a heritable bone disorder that is characterized by bone fragility and often caused by mutations in the Type I collagen-encoding genes, *COL1A1* and *COL1A2*. The pathophysiology of OI, particularly at the cellular level, is still not well understood. In bone, mesenchymal stem cells (MSCs) and osteoblasts (Obs) exert their function, at least partially, through the secretion of extracellular vesicles (EV). EVs are a heterogeneous group of nanosized membrane-enclosed vesicles that carry/transfer a cargo of biomolecules from the secreting cell to its target cells. Our objective was to characterize EVs secreted by human control (HC)- and OI-MSCs and their derivative Obs, with focus on their protein content. We hypothesize that there will be differences in the protein content of EVs secreted by the OI-Obs compared to the HC-Ob, which may indicate a deviation from healthy Ob behavior and; thus, a role in OI pathophysiology.

Methods: MSCs were isolated from three adipose tissue samples harvested from two *COL1A1*-OI and one HC patients. They were proliferated in an EV-depleted media, then induced to differentiate to osteoblasts, which then formed and mineralized the extracellular matrix (ECM). EVs secreted by the MSCs (MSC-EV) and the Obs (Ob-EV) were then enriched by a combined technique of ultrafiltration and differential ultracentrifugation. Liquid chromatography- tandem mass spectrometry of the EV proteins was then done.

Results: Proteomic analysis revealed a total of 384 unique proteins in all the EV groups combined, of which 373 (97%) were found in Vesiclepedia; an EV database. This indicated a good enrichment of our samples with EV proteins and thus validates the nature of our sample to be rich in EVs. 67 proteins of the total 384 were exclusively or significantly upregulated (p-value ≤ 0.05) in OI-Ob-EV and 28 proteins in the HC-Ob-EVs, relative to each other. We observed that

there were differences in the cellular origin of the identified proteins, which indicates differences in the biogenesis and heterogeneity of their packaging EVs. Molecular function and biological process analyses of the HC-Ob-EV proteins showed, as expected, predominantly calcium-related activities such as extracellular matrix (ECM) mineralization. On the other hand, OI-Ob-EV proteins were still predominantly exhibiting ECM organization, formation and disassembly functions. Several annexins were differentially and significantly upregulated in the HC-Ob-EVs. ECM proteins, e.g. fibronectin, fibulins, and laminins, and proteases/proteinases, e.g. HtrA1, matrix metalloproteinases-14, cathepsin B, were differentially and significantly upregulated in the OI-Ob-EVs.

Conclusion: The persistent expression of ECM proteins might indicate the presence of an immature ECM that the OI-Obs are still trying to organize. This may be in part due to the upregulation of proteases/proteinases causing§ ECM degradation/resorption.

ECM mineralization is largely dependent on the presence of an organized mature ECM, and this being compromised in the OI environment, may be a contributor to the bone fragility seen in these patients. Annexins, which are calcium-binders that are vital for various processes in ECM mineralization, were significantly downregulated in the OI-Ob-EVs and this may be a further contributor to ECM mineralization impairment and bone fragility.

Résumé

Introduction : L'ostéogénèse imparfaite (OI) est une maladie héréditaire osseuse caractérisé par une fragilité d'os et qui est souvent un résultat d'une mutation de gène du collagène type I, *COL1A1* et *COL1A2*. La pathophysiologie de l'OI, particulièrement au niveau cellulaire, reste toujours mal compris. Les cellules souches mésenchymateuses (CSM) et les ostéoblastes (Ob) dans l'os exercent leur fonction du moins en partie par la sécrétion des vésicules extracellulaires (VE). Les VE sont un group hétérogène des vésicules nanométriques enfermées qui transportent des biomolécules d'une cellule de sécrétion vers des cellules cibles. Notre objectif était de caractériser les VE sécrétaient par des contrôles humains (CH)- et les CSM de l'OI et leurs dérivés de Obs, en mettant l'accent sur leur teneur en protéines. Notre hypothèse est basée qu'il y aura des différences dans la teneur protéine des VE sécrété par les Ob de l'OI par rapport aux Ob des CH, qui pourrait nous indiquer une déviation de Ob normal, ainsi un rôle dans la pathophysiologie de l'OI.

Méthodes : Les CSM ont été isolé des échantillons de trois tissu adipeux fournis par deux patients de l'OI avec une mutation de COL1A1, et un patient CH. Elles étaient proliférées dans un média sans VE, ensuite induite pour se différencier à des Obs, qui ont été formé et qui ont minéralisé la matrice extracellulaire (ME). Les VE sécrété par des CSM (VE-CMS) et les Ob (VE-Ob) ont été ensuite enrichi par une technique d'ultrafiltration et d'ultracentrifugation. La chromatographie liquide et spectroscopie de masse des protéines des VE ont été performé après.

Résultats : une analyse protéomique a démontré 384 protéines uniques dans tous les groups de VE ensemble, parmi 373 (97%) qui ont été trouvé sur Vesiclepedia ; une base de données des VE. Ceci indique la richesse en protéine de VE dans nos échantillons et qui valide la nature de

notre échantillon qui est riche en VE. 67 protéines du totale de 384 ont été exclusivement ou considérablement régulée à la hausse (valeur de $p \leq 0.05$) chez les Ob de l'OI et 28 protéines chez les Ob du CH, par rapport à l'autre. Nous avons observé des différences dans l'origine cellulaire des protéines identifiées, qui indique des différences dans la biogénèse et de l'hétérogénéité de leur VE. La fonction moléculaire et l'analyse protéine du processus biologique de VE-Ob du CH a démontré comme prévu, des activités reliées au calcium tel que la minéralisation de la matrice extracellulaire (ME). En revanche, les protéines du VE-Ob de l'OI ont principalement présenté l'organisation du ME, la formation et les fonctions du désassemblage. Plusieurs annexines étaient d'une manière différentielle et considérablement régulée à la hausse chez les VE-Ob du CH. Les protéines de ME, par exemple fibronectine, fibulines et laminines et des protéases/protéinases, par exemple HtrA1, metalloproteinases-14 matrice, cathepsine B, ont été de manière différentielle et considérablement régulée à la hausse chez les VE-Ob de l'OI.

Conclusion: L'expression constante des protéines de ME pourrait indiquer la présence de ME immature que les Ob-OI essayent d'organiser. Ceci pourrait d'une partie être dû à la régulation positives des protéases/protéinases qui causent la dégradation/résorption de ME.

La minéralisation de ME est dépendante en grande partie sur la présence organisé des matures ME et ceci est compromise par l'environnement dans l'OI, qui pourrait contribuer à la fragilité osseuse qui observé chez ces patients. Annexines, qui sont des liants de calcium et essentiel dans plusieurs processus de minéralisation de ME, étaient considérablement régulée à la baisse chez les VE-Ob de l'OI qui pourrait contribuer à l' insuffisance de la minéralisation de la ME et la fragilité osseuse.

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Contribution of Authors

Monther Abuhantash was the primary author of this thesis reviewed by Dr. Hadil Al-Jallad and with constant input from Dr. Reggie Hamdy.

All protocols were optimized by Dr. Hadil Al-Jallad.

All experiments were designed by Dr. Hadil Al-Jallad, and were performed by Monther Abuhantash.

Monther Abuhantash analyzed all the data presented in this thesis under the supervision of Dr. Hadil Al-Jallad with final input from Dr. Reggie Hamdy.

Nanoparticle tracking analysis was performed by Monther Abuhantash with technical support from Laura Montermini at Dr. Rak's laboratory.

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Isolation and characterization of the adipose tissue-derived mesenchymal stem cells was done by Dena Bakhsh and Mayumi Umebayashi.

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Abbreviations

A Disintegrin and metalloproteinase domain-containing protein-10: ADAM10

A Disintegrin and Metalloproteinases with Thrombospondin motifs 2: ADAMTS2

Actin cytoplasmic 2: ACTG1

Adipose tissue: AT

Alizarin Red S: ARS

Alkaline Phosphatase: ALP

Alpha-1 chain of type-I collagen: COL1A1

Alpha-2 chain of type-I collagen: COL1A2

Basigin: BSG

Bone marrow: BM

Bone Morphogenetic Protein 1: BMP1

Bone-restricted interferon-induced transmembrane protein-like: BRIL

Caveolae-associated protein 1: CAVN1

Caveolin-1: CAV1

Dynamic Light Scattering: DLS

EH domain-containing protein 1: EHD 1

EH domain-containing protein 2: EHD 2

Endoplasmic reticulum: ER

Endosomal sorting complexes required for transport: ESCRT

Extracellular mineralization: ECM

Extracellular Vesicle: EV

Fetal Bovine Serum: FBS

Fibronectin: FN

Flotillin-1: FLOT 1

Flotillin-2: Flot-2

Gene Ontology: GO

Guanine nucleotide-binding protein, alpha inhibiting activity polypeptide 3: GNA I3

Guanine nucleotide-binding protein, alpha inhibiting activity polypeptide 2: GNA I2

Guanine nucleotide-binding protein, alpha stimulating activity polypeptide1: GNA S1

Heat Shock Protein 47: HSP47

Heat Shock Protein 70: HSP70

Heat Shock Protein 90: HSP90

Heat Shock Protein A8: HSPA8

Hematoxylin and Eosin stain: H&E stain

Heparan Sulfate Proteoglycan: HSPG

High-Temperature Requirement A1: HtrA1

Human Control: HC

Integrin alpha-V: ITG AV

Integrin Subunit Alpha 3: ITG A3

Integrin Subunit Beta 5: ITG B5

Integrin, Alpha 4: ITG A4

Interferon Induced Transmembrane Protein 5: IFITM5

International Society for Cellular Therapy: ISCT

International Society of Extracellular Vesicles: ISEV

Intraluminal Vesicles: ILV

Lysosome-associated membrane glycoproteins-1: LAMP1

Lysosome-associated membrane glycoproteins-2: LAMP2

Matrix metalloproteinase-14: MMP14

Matrix Vesicle: MV

Mesenchymal Stem cell: MSC

Multivesicular Bodies: MVB

Nanoparticle Tracking Analysis: NTA

Osteoblast: Ob

Osteogenesis Imperfecta: OI

Osteoprotegerin: OPG

Pigment epithelium-derived factor: PEDF

Platelet-derived growth factor: PDGF

Programmed Cell Death 6 Interacting Protein: PDCD6IP

Receptor activator of nuclear factor κ B Ligand: RANKL

Receptor activator of nuclear factor κ B: RANK

Resistive pulse sensing: RPS

Tartrate-Resistant Acid Phosphatase: TRAP

Transforming growth factor beta: TGFβ

Tumor Susceptibility Gene 101: TSG101

Vascular endothelial growth factor: VEGF

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Chapter 1: Background

Section 1: The Skeleton

1.1.1 Overview

The human skeleton is a complex system composed of bone, muscles, tendons, ligaments and cartilage that serve a variety of functions all over the body. Bone is integral for structural support, protection of internal organs and locomotion by functioning as a muscle attachment site (1). It is also the main center for regulating mineral hemostasis, acid-base balance and hematopoiesis in the enclosed bone marrow (2).

There are two histological types of bone that make up the human skeleton system: cortical and trabecular bone (1). Cortical bone is the solid, dense, outer part of the skeletal structure, which confers bone mechanical strength and protection. It is organized in what is called a Haversian system or osteons, which consist of a canal containing a blood vessel surrounded by lamellae. On the other hand, trabecular bone, also called cancellous or spongy bone, is composed of a relatively thin meshwork found in the inside of long bones and the inner portions of flat bones. It is more elastic and has higher bone turnover rate as it is responsible for the majority of remodeling that occurs in the bone's lifetime; this is important for maintaining its integrity as well as executing its metabolic functions. The ratio of cortical to trabecular bone varies depending on the bone itself and its skeletal site; however, cortical bone comprises 80% of the overall skeletal system mass and trabecular bone comprises the remaining 20%. Bone could also be classified by its mechanism of formation during development.

1.1.2 Endochondral and intramembranous ossification

In the embryological stage of development, bone is formed by one of two mechanisms: endochondral or intramembranous ossification, as will be detailed below (3). Endochondral ossification occurs in the base of the skull, posterior aspect of the skull, axial skeleton and the appendicular skeleton. Intramembranous ossification occurs in the remaining bones of the body, which are the membranous viscerocranium and neurocranium, and part of the clavicle. Skeletogenesis is initiated by the migration of mesenchymal cells derived from various embryonic lineages to the sites of the future bones.

In endochondral ossification, mesenchymal stem cells differentiate into chondrocytes, which lay out a cartilage matrix/model where bone is to be formed. This cartilage template is surrounded by the periosteum/perichondrium, which forms the boundary between the developing bone and the surrounding soft connective tissue. Various cells then invade this cartilage matrix from the perichondrium (3). The cartilage gets calcified and then absorbed by osteoclasts and replaced with mineralized bone by osteoblasts. Blood vessels also infiltrate the cartilaginous matrix to bring in osteoclast- and osteoblast-precursors, which form the primary ossification centers (4). As bone development continues, the primary ossification center expands to form secondary ossification centers at the epiphyseal growth plates on each ends of the bone, which are then responsible for longitudinal bone growth (5, 6).

In intramembranous ossification, embryological mesenchymal cells migrate to the site of the future bone that is to be formed. They condense to form a highly vascular connective tissue membrane and a diffuse network of bony spicules. Osteoblasts then aggregate/condense and deposit a type I collagen-rich extracellular matrix called the osteoid, which gets mineralized (7). The osteoblasts that get encased within the mineralized osteoid are now called osteocytes, and

although have decreased metabolic activity relative to osteoblasts, they still produce matrix proteins (8).

1.1.3 Bone Remodeling

During childhood and adolescence, bone growth and development occur at the growth plates of the metaphyseal and epiphyseal areas. Bone modeling is the process by which a bone's shape and size are formed to ensure capability of withstanding mechanical loading forces subjected to the skeleton (1). Bone remodeling is a continuous process in which there is constant resorption of old bone that gets replaced with new healthy bone. This aims to prevent progression of bone microdamage/microfracture into larger deformities (1). It is mediated by the coordinated actions of osteoblasts and osteoclasts through four sequential phases: 1) activation, 2) resorption, 3) reversal and 4) formation phases. The activation and resorption phases are when osteoclasts are recruited to resorb bone, reversal phase is when osteoblasts are recruited and formation phase is when osteoblasts mineralize the bone matrix (1).

1.1.4 Bone Composition

Bone tissue is composed of hydroxyapatite mineral, collagen, non-collagenous proteins, water, lipids, vessels and cells. These elements exist in varying amounts between individuals depending on their age, gender, bone site, health and dietary status. The main cells found in the bone environment are: osteoblasts, osteocytes, osteoclasts, bone-lining cells, mesenchymal stem cells and hematopoietic stem cells. They are responsible for maintaining the integrity of bone structure and for executing its functions.

Bone is composed of numerous lamellae, each consisting of spirally oriented collagen fibrils all lying in the same direction within each lamellae, but oriented in a different direction in the adjacent one; thus, contributing to the biomechanical stability characteristic of bone (9).

Bone matrix proteins are composed of 85-90% collagenous proteins, which are mostly type 1 with trace amounts of type III and V (1). Type 1 collagen confers bone strength and gives it its mechanical properties, while type III and V regulate collagen fibril assembly and diameter. The remaining 10-15% of the matrix proteins are non-collagenous, and could be broadly divided into proteoglycans, glycosylated proteins, glycosylated proteins with cell-attachment activities and γ–carboxylated proteins (1). The most interesting glycosylated protein found in bone matrix is alkaline phosphatase (ALP). As will be further elaborated at a later section, ALP has a vital role in the process of osteogenesis and bone matrix mineralization. Fibronectin is a glycoprotein that is considered one of the earliest matrix proteins (10) and, as well be detailed later, serves an important role in extracellular matrix formation (11, 12).

1.1.5 Mesenchymal stem cells and its sources

Mesenchymal stromal cells are spindle-shaped plastic-adherent cells that can be isolated from the bone marrow (BM), adipose tissue (AT) as well as other tissues (13-15). However, there is some confusion and disagreement over the use of the right nomenclature to describe these cells as they are often inappropriately labeled as stem cells (16). For this reason, the international society for cellular therapy (ISCT) has suggested that these cells can only be labeled as Mesenchymal Stem Cells (MSCs) once their self-renewal and multipotency is proven *in-vitro* (13, 16). In addition, they proposed minimal criteria to characterizing MSCs by their: 1) morphology, 2) multilineage differentiation potency and 3) immunophenotype (16, 17). MSCs have a fibroblast-like morphology with plastic-adherence in tissue culture. They are capable of

differentiating into at least three cell lines: osteoblastic, adipocytic and chondrocytic lineages. Immunophenotypically, they express CD105, CD73, CD90, CD44, CD34 but are negative for HLA-DR, CD31, CD45, CD235a, CD11b, CD79 or CD19.

MSCs from the different sources were shown to have similar morphological and immunophenotypical characteristics but dissimilar differentiation potentials (14, 18). For instance, MSCs from the BM, AT and umbilical cord differentiated into osteoblasts and chondrocytes similarly, as demonstrated by ECM mineralization and mucopolysaccharide production respectively (18). Bone marrow- and adipose tissue-derived MSCs had a greater propensity to differentiate into adipocytes than did umbilical cord-derived MSCs under similar culture conditions (18). However, in terms of practicality and accessibility, adipose tissue is superior to the other sources, particularly the BM (19-21). It is relatively more accessible, and a minimally invasive harvesting procedure yields a greater supply of MSCs (19-21). This has made them the ideal source of MSCs for bone disease studies, for bone regeneration and other therapeutic purposes.

1.1.6 Bone matrix mineralization

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), the most abundant component of bone, is a mineral crystal that is mainly composed of calcium- and hydroxide-deficient apatite and carbonate (22). Bone matrix mineralization is the physiological process by which these calcium phosphate mineral crystals precipitate on type I collagen-rich bone matrix. It is still unclear on the exact mechanism on what initiates bone mineralization, but several studies have reported that matrix vesicles (MV) may have an important role as the nucleation sites where mineralization first appears (23, 24). MVs are membrane-bound/anchored vesicles that bud off from the surface membrane of osteoblasts and are located within the extracellular matrix of bone (25). They act as

an enclosed environment where mineralization can occur protected from the surrounding areas where mineralization is normally inhibited (7). They contain a nucleation core that is rich in alkaline phosphatase, annexins, type III sodium-phosphate (Na/Pi) co-transporter and a cytosolic phosphatase (PHOSPHO1) (24-26). ALP is also concentrated on the membranes of these matrix vesicles (27, 28) and function by hydrolyzing inorganic pyrophosphate to inorganic phosphate; the former is an inhibitor of hydroxyapatite crystal formation (29). Therefore, by removing this inhibition and providing inorganic phosphate within MVs, ALP acts as one of the key regulators of bone matrix mineralization. It is also for this reason that ALP is considered the marker of choice when assessing the developmental maturity of mineralized tissue. Type III sodium-phosphate (Na/Pi) co-transporter and PHOSPHO1 also provide inorganic phosphate (27). The annexin proteins are calcium-binders that promote the influx of calcium through their channels into the MVs (26). By incorporating calcium with phosphate, hydroxyapatite crystals are formed within these vesicles then propagate through their membrane into the extracellular matrix, filling the spaces between the collagen fibrils (25, 27).

1.1.7 Type I collagen

Type I collagen is the main protein component of the extracellular matrix of bone, skin and other connective tissues, and is mainly produced by osteoblasts (30). Despite it being a relatively simple structure, collagen's biosynthesis is complex with many steps involving its regulation, modification, folding, transport and secretion, as will be summarized below (31, 32) (Figure 1.1). This delicate manufacturing process has been extensively studied and characterized, which expanded our knowledge of how various impairments in the normal process of synthesizing such a simple structure can give rise to a number of different diseases.

Type I collagen is a triple helix heterotrimer composed of 3 polypeptide chains, two α1 and one α2 chains, translated from *COL1A1* and *COL1A2* genes respectively. Proα1 and proα2 chains are each composed of three distinct domains: an amino-terminal (N-propeptide), a carboxy-terminal (C-propeptide), and in between them, a glycine-rich polypeptide domain. This central collagenous domain is composed of Gly-Xaa-Yaa repeats, where the X position is usually a proline residue and the Y position is either hydroxyproline or hydroxylysine residues. These type I procollagen polypeptide chains are synthesized/translated in the rough endoplasmic reticulum, where they also undergo extensive modifications prior to forming the triple helix collagen structure.

Co-translation, hydroxylases at the endoplasmic reticulum modify proline residues to hydroxyproline, particularly at the Y position of the Gly-Xaa-Yaa repeats. This modification is vital in providing thermal stability to the collagen triple helix (33, 34). Lysine residues that are found at the Y position also undergo hydroxylation to hydroxylysine residues, which then undergo subsequent glycosylation. Next, a triple helix of three procollagen α chains is formed via interchain and intrachain disulfide bonds, starting from the carboxy-terminal end to the amino-terminal end. Proper assembly is thought to be dependent on the presence of a glycine residue at every third position in the collagen domain. The newly-formed triple helix is then stabilized by SERPIN H1 (also known as HSP47, encoded by SERPINH1) and FKBP65 (35, 36). FKB65 increases thermal stability and prevents the premature association of type I procollagens molecules. Following folding of the procollagen into its helical form, SERPINH1 controls the quality of the formed collagen molecules, then transports them from the rough endoplasmic reticulum to the Golgi apparatus. The procollagen is then released in secretory vesicles to the extracellular matrix. There, two proteases: A Disintegrin and Metalloproteinases

with Thrombospondin motifs 2 (ADAMTS2) and Bone Morphogenetic Protein 1 (BMP1), recognize specific sequences at both ends of the procollagen, and then cleave off the N- and C-terminal propeptides to form the mature form of type 1 collagen protein. The mature collagen molecules then spontaneously self-assemble into collagen fibrils (fibrillogenesis). Lysyl oxidase then stabilizes these collagen fibrils by forming covalent lysine-hydroxylysine cross-linkage.

Fibronectin (FN) and their binders, integrins, set the site of where collagen assembly takes place in the extracellular matrix (12). Seminal studies showed that antibodies against the collagen-binding site on FN resulted in the inhibition of collagen fibril assembly; thereby, supporting the hypothesis that collagen fibrillogenesis is dependent on the presence of FN (37).

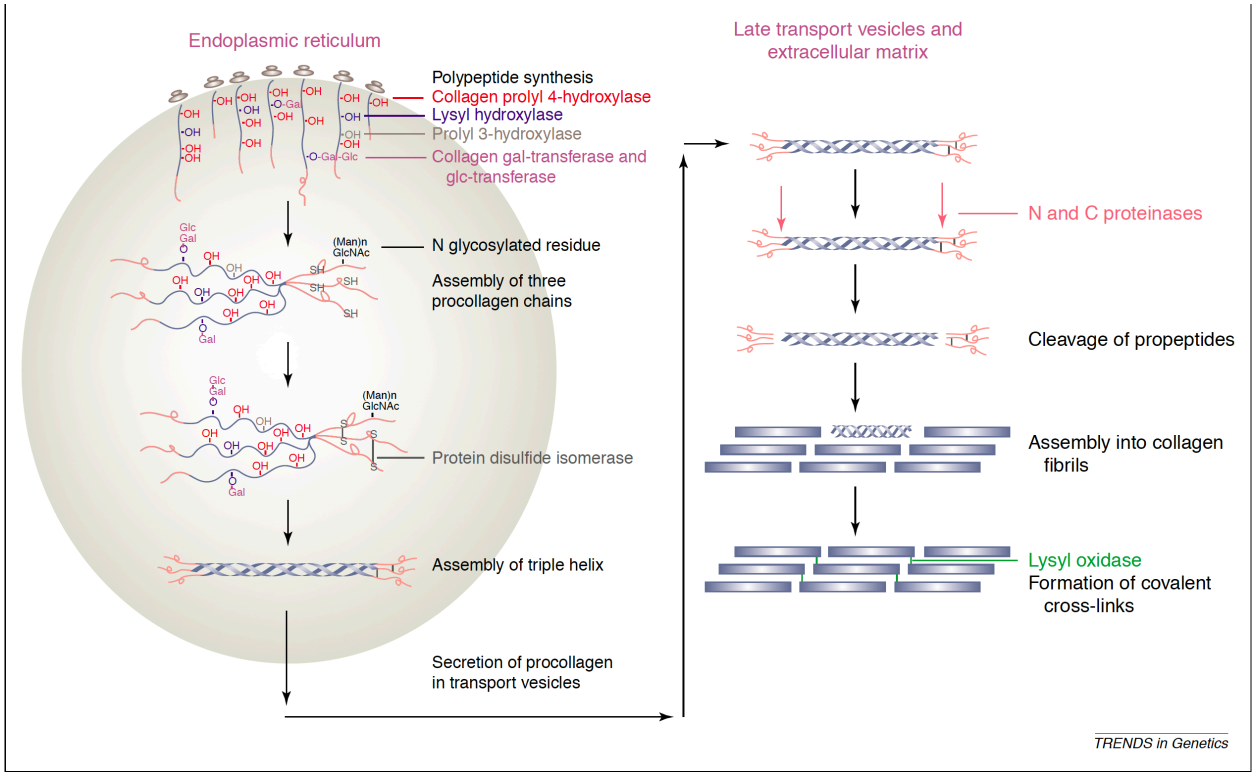

Figure 1.1:Overview of the biosynthesis of type I collagen

Schematic representation of the biosynthesis of procollagen chains and their secretion into the extracellular space, where they assemble into fibrils. This figure was reproduced from Myllyharju et al. (31) with permission.

Section 2: Osteogenesis Imperfecta

1.2.1 Overview

Osteogenesis imperfecta is widely known as 'brittle bone disease'; however, it is a phenotypically heterogeneous systemic disease affecting skeletal and extra-skeletal tissues. It is relatively rare, occurring in approximately 1 in 15,000-20,000 births, most of which are autosomal dominant in inheritance (30).

Osteogenesis Imperfecta was first reported in 1979 by Sillence et al, where it was described as an inheritable disease compromising the integrity of bone, sclera and skin of affected individuals (38). Sillence et al. also proposed a classification system for OI based on its clinical presentation, radiographic features and inheritance pattern, which was considered the standard for over two decades. With extensive genetic studying and an increased understanding of OI, an adaptation of this classical classification has been proposed taking into consideration newly discovered involvement of recessive genes in OI pathogenesis.

1.2.2 Genotype and pathogenesis

OI types I to IV are caused by different mutations in the genes that code for α1 and α2 chains of type 1 collagen, which are *COL1A1* and *COL1A2* genes respectively (39). However, these OI types differ in that their causative mutations result in different effects on type 1 collagen and thus, variable phenotype.

Type I is caused by mutations that translate into premature termination codons in the *COL1A1* transcript (null *COL1A1* allele) (40). This causes a decrease in the production of structurally normal type 1 procollagen and subsequent extracellular matrix insufficiency.

In approximately 80% of OI types II to IV cases, the cause is a glycine substitution mutation in the *COL1A1* or *COL1A2* genes, with a splice-site mutation responsible for the

remaining 20% (39). This results in an interference with the collagen helix folding process which compromises its secretion and/or processing; therefore, causing it to undergo post-translational over-modification (39, 41). There are variable fates for these misfolded collagen molecules. They could get retained in the endoplasmic reticulum (ER) causing intracellular stress. This can impair the osteoblast's differentiation or may result in its autophagy due to the activation of ER-associated or lysosomal degradation systems (42-44). Alternatively, these structurally abnormal collagen molecules may still get secreted into the extracellular space resulting in a heterogeneous extracellular matrix that is a mixture of normal and mutated collagens. The resultant outcome is a dysfunctional extracellular matrix and a decreased normal collagen synthesis (30).

Another autosomally dominant form of the disease is OI type V. However, this type is not caused by mutations in either *COL1A1* or *COL1A2* genes. It is caused by mutations in *IFITM5* (Interferon Induced Transmembrane Protein 5), which codes for BRIL (bone-restricted interferon-induced transmembrane protein-like) protein. BRIL protein has been shown to be vital for bone mineralization, as seen in some animal models in which BRIL overexpression resulted in increased mineralization and conversely, knockdown of BRIL in MC3T3 osteoblasts resulted in reduced mineralization (45).

As previously mentioned, autosomal recessive types of OI exist. They occur due to mutations in genes which code for proteins involved in type 1 collagen biosynthesis, processing, crosslinking and posttranslational modification, as well as those involved in osteoblast differentiation and function (30). For instance, pigment epithelium-derived factor (PEDF), also known as serpin F1 (SERPINF1), is encoded by SERPINF1 gene, which is the culprit in OI type VI pathogenesis (46, 47). PEDF is responsible for up regulating osteoprotegerin, which is an inhibitor of osteoclast maturation, differentiation and proliferation (48, 49). Therefore, the loss of

function mutation of SERPINF1 results in an increase in the number of activated osteoclasts thus promoting bone resorption and ultimately causing OI type VI (48, 49). The rest of the autosomally recessive OI types are highlighted briefly in the below Figure 1.2.

Osteogenesis imperfecta type	Inheritance	Phenotype	Gene defect
Classical Sillence types			
I	AD	Mild	Null *COL1A1* allele
II	AD	Lethal	*COL1A1* or *COL1A2*
III	AD	Progressive deforming	*COL1A1* or *COL1A2*
IV	AD	Moderate	*COL1A1* or *COL1A2*
Unknown etiology			
V	AD	Distinctive histology	Unknown
Mineralization defect			
VI	AR	Mineralization defect, distinctive histology	*SERPINF1*
3-hydroxylation defects			
VII	AR	Severe (hypomorphic) Lethal (null)	*CRTAP*
VIII	AR	Severe to lethal	*LEPRE1*
IX	AR	Moderate to lethal	*PPIB*
Chaperone defects			
X	AR	Severe to lethal	*SERPINH1*
XI	AR	Progressive deforming (Bruck syndrome 1)	*FKBP10*
Unclassified osteogenesis imperfecta-like or collagen-based disorders			
Bruck syndrome 2	AR	Joint contractures	*PLOD2*
Caffey disease	AD	Cortical hyperostosis	*COL1A1*
Osteoblast maturation defects	AR	Moderate	*SP7*

Abbreviations: AD, autosomal dominant; AR, autosomal recessive.

Figure 1.2: OI types, inheritance, phenotype and genotype

Schematic representation of the different types of osteogenesis imperfecta, with their respective mode of inheritance, phenotypes and genotypes. This figure was obtained from Forlino et al. (50) with permission.

1.2.3 Phenotype

Osteogenesis Imperfecta often has a widespread and variable presentation. This is mostly due to the fact that collagen is the most abundant protein in the human body found across many different tissues (51). However, bone remains the most commonly affected tissue, specifically with low bone mass and fragility (52).

OI type I has the mildest phenotype, and is characterised by a triad of fractures at the time of ambulation, blue sclera and hearing loss, but little to no bone deformities or abnormal dentition (50). OI Type II infants are born with severe bone deformities, a soft cranium and thorax skeletal immaturity (50). It is also perinatally lethal, most commonly as a result of scoliosis and rib cage deformities causing loss of pulmonary function and respiratory failure (53). Type III is the most severe non-lethal type, where affected individuals suffer from hundreds of fractures throughout their lifetime as well as extreme short stature, characteristic facial deformities, vertebral compression and scoliosis (50). On the other hand, type IV OI has a variable severity with a broad phenotypic range from mild to severe. Cardiac and neurological complications can also occur in severe OI cases, such as valve regurgitation and brain herniation, which often cause serious and significant morbidity and mortality (54, 55).

1.2.4 Diagnosis

Initial/preliminary diagnosis of osteogenesis imperfecta is done by clinical and radiographic examination (56). The diagnosis is usually made prenatally, at birth or in early childhood at the time of ambulation.

Low-energy bone fracture(s), bowed bones and short stature are the most common manifestations that prompt the clinician to further investigate the child using skeletal

radiographs, dental examination and ophthalmic evaluation. Skeletal radiographs often reveal generalized osteopenia and skeletal manifestations of the disease. Other bone quality studies that could be useful in investigating OI include Dual-energy X-ray Absorptiometry (DEXA) scan and bone histomorphometry (30).

Because OI has unspecific and variable disease features, its diagnosis can only be confirmed with genetic testing. All the genes that are known to be associated with OI are screened for, starting with the most likely culprits, the COL1 genes. Some studies have reported the possibility of assessing certain protein levels in patients to diagnose or screen for osteogenesis imperfecta, e.g. PEDF serum levels to diagnose type VI osteogenesis imperfecta (48). However, there is still a lack of a reliable and accurate serum protein marker to screen for or diagnose OI.

1.2.5 Medical Management

To date, there is no known treatment, medicine or surgery that can cure OI. Patient management is provided by a multidisciplinary team, whose primary focus revolves around maximizing the patient's quality of life, independence and self-care. For this reason, physical rehabilitation is universally provided to all OI patients to counter the effects of weak muscles, fragile bones and loss of gross motor skills (57). This is important to attain domestic skills necessary for sufficient self-care and home independence, as well as to aid patients in ambulation, especially those with the mild form of the disease.

Bisphosphonates are considered the gold standard of pharmacological management of OI. They are synthetic analogues of pyrophosphates and work by inhibiting osteoclasts resulting in their apoptosis. The reasoning behind their use is to decrease bone resorption and thus counteract the high bone turnover that occurs in OI (58). They allow the continued production of type 1

collagen that is quantitatively or qualitatively defective in OI. It is hypothesized that this is beneficial to the bone strength due to the resultant increase in bone volume even if it is of impaired quality (30). Conversely, several meta-analysis reported that bisphosphonates do not significantly reduce the overall rate of fractures in OI (59, 60) nor improve growth, ambulation, muscle strength or pain (61). Animal studies done to assess the long-term effects of osteoclast inhibition showed a resultant non-dynamic bone where microcracks/microfractures go unrepaired and so propagate (62, 63). This persistent inhibition of bone turnover paradoxically resulted in a decrease in mineralization heterogeneity further aggravating bone brittleness in these animal models. The maximum benefit of bisphosphonates on bone histology and bone mineral density occurs after 3 years of use (61, 64); however, physicians use them until skeletal maturity occurs in the child despite the absence of any reported benefit of doing so.

Alternative pharmacological drug therapies exist, but their superiority over bisphosphonates and safety profile are yet to be fully determined. Denosumab is an antibody against RANKL preventing it from attaching to the RANK receptor. Thus, it mimics the inhibitory actions of OPG in the bone remodeling system by promoting antiresorptive effects (65). Antisclerostin is a monoclonal antibody that alleviates the effects of sclerostin in the regulation of bone formation; thereby, promoting osteoblast function without antiresorptive adverse effects (66).

1.2.6 Surgical management

Bone fractures and bowing are common features of OI that need orthopedic surgical intervention to allow for restoration of ambulation and self care. The surgical technique consists of an osteotomy (a cut in the bone) to correct the bone alignment, which is then stabilized with

an intramedullary rod (67). However, surgical correction is associated with a high re-operative rate of ≈50-80% (68), which is in part caused by the inadequate healing that very frequently occurs at the osteotomy site (69, 70). The revision surgery is often complex, with immense burden on the patients, their families and the healthcare system.

1.2.7 Knowledge gap

OI is a heritable disorder whose genetic causes are well established. However, it is still not quite well understood how the causative mutations translate and affect cellular behavior to result in its skeletal features. Therefore, it would be logical to fill this knowledge-gap by studying bone cells of OI patients, particularly the bone-forming osteoblasts. Interestingly, osteoblasts and other bone cells interact with each other and their surrounding environment through paracrine signaling (71, 72), which could be studied to advance our understanding of OI osteoblasts and pathogenesis.

Section 3: Extracellular Vesicles

1.3.1 Definition, content and significance

Extracellular Vesicles (EVs) is a term that collectively refers to a heterogeneous group of nanosized lipid membrane-enclosed vesicles secreted by cells into their extracellular environment. They are involved in some intercellular communications by carrying/transferring a cargo of biomolecules from a secreting cell to its target cell(s); thereby; influencing its function (73-75). They contain proteins, lipid and nucleic acids, with the specific content depending on the type of the secreting cell and its metabolic status at the time of secretion (76-78). EVs have diverse biological effects on recipient cells, which is due to secreting cells releasing heterogeneous EV groups each with their unique content and function (79). This was shown when a study assessing breast cancer cells and their vesicular secretions noted differential packaging of miRNAs into distinct EV subpopulations (78).

Secreted EVs could be found in various bodily fluids including the blood, saliva and urine, and could also be isolated from a tissue culture medium (76, 77). These findings increase EVs' practicality and obtainability to investigate their potential role in physiological and pathological processes.

One of the biggest challenges faced by the EV field is the presence of multiple terms assigned to describe them in the literature (80). Throughout the years, researchers in the EV field have been designating names to secreted vesicles depending on their area of interest and the secreting cell. This has led to a messy mix-up of names describing, what are in essence overlapping EV populations. Hence, this lack of consensus on EV nomenclature prompted the International Society of Extracellular Vesicles (ISEV) to choose the general term of "Extracellular Vesicles" to encompass all of these vesicles, until more precise guidelines and

terminology are established (80). Having said that, there are several main types of EVs that have been commonly described and categorized based on their discrete biogenesis (81-83). They are: exosomes, microvesicles and apoptotic bodies (Figure 1.3).

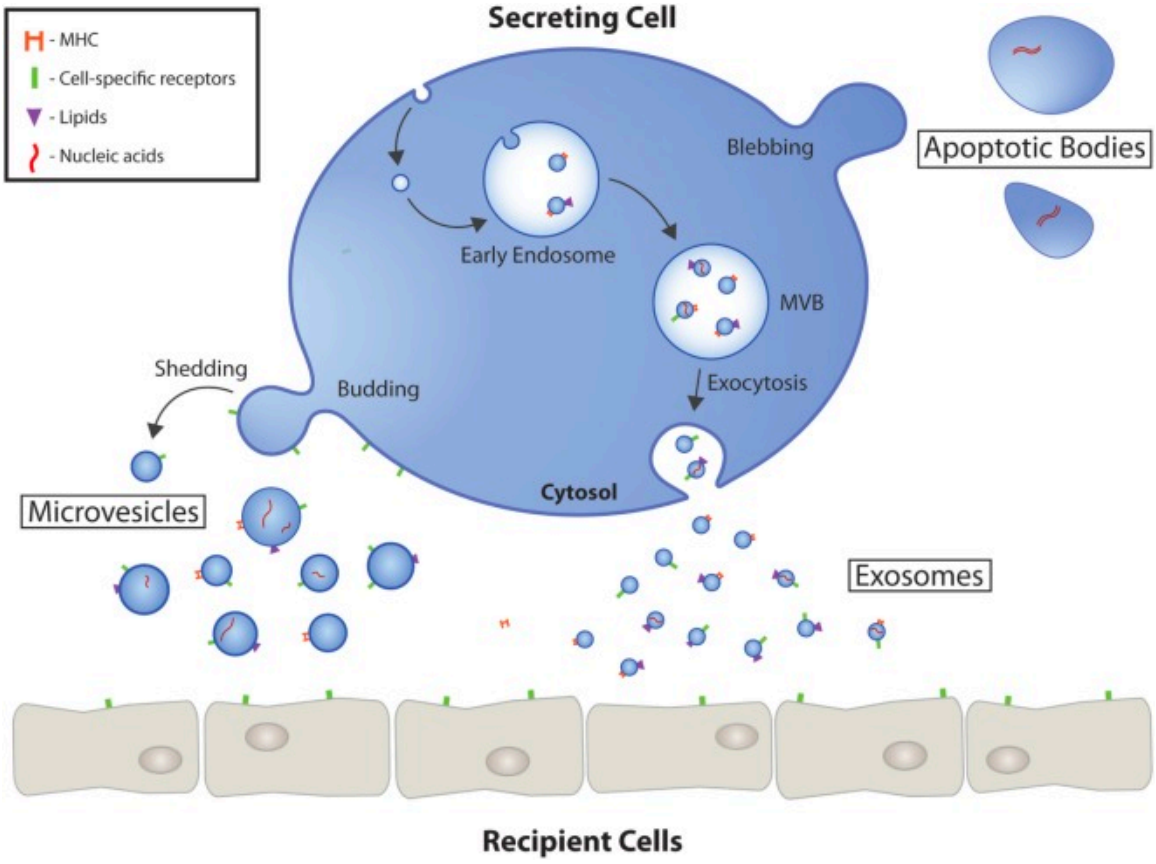

Figure 1.3: Biogenesis of the main types of EVs

Diagram briefly illustrating the discrete biogenesis of the three main types of EVs: exosomes, microvesicles and apoptotic bodies. It also shows how the secreting cell, through EVs, transfers various biomolecules to its recipient cells. The figure was adapted from Gustafson et al. (84).

1.3.2 Types of EVs and their biogenesis

The three main subtypes of EVs have a common feature of a lipid bilayer surrounding a cargo of biomolecules, whereas they differ in their size range, floating density and biogenesis (83, 85). However, these differences in properties are considered insufficient to make clear distinctions amongst the EVs subtypes (82, 86).

Exosomes are about 30-150nm in diameter and, as will be described below, originate internally through the endocytic pathway (83, 87). Endosomes are membrane-bound intracellular sorting organelles that form from the internalization of extracellular ligands or cellular components. As they mature, endosomes form intraluminal vesicles (ILV). ILVs, found within multivesicular bodies (MVB), contain proteins, lipids and nucleic acids. ILVs can then either be degraded by lysosomes, or can be recycled by fusing with the cell's plasma membrane then releasing their content into the extracellular milieu as exosomes (exocytosis). Endosomal sorting complex required for transport (ESCRT) system is composed of four protein complexes that are integral to the formation of ILVs and MVBs, and exocytosis (88).

Conversely, microvesicles, also known as ectosomes or microparticles, have a relatively larger size range of 100-1000nm in diameter and are formed by budding off the secreting cell's plasma membrane (83, 89). Microvesicles' formation is induced by the translocation of phosphatidylserine to the outer membrane leaflet of the plasma membrane (90). The budding process occurs as a result of translocases-regulated asymmetrical phospholipid redistribution within the plasma membrane. The secretion process is then facilitated by cytoskeletal actin-myosin contracture at the neck of the vesicle (91).

Apoptotic bodies are the largest of the main EV groups, ranging in diameter from 50-5000nm, and are released in a coordinated programmed process from cells undergoing apoptosis

(92). Due to their large size, they often contain tightly-packed DNA fragments and organelles (93).

1.3.3 EVs enrichment and concentration

The International Society of Extracellular Vesicles (ISEV) recently published a position statement with the latest recommendations and guidelines for studying extracellular vesicles (94). Of the many points they highlighted was the consensus that, irrelevant of the source, EVs cannot be completely isolated from a biological fluid or cell culture medium, and absolute EV purification is unrealistic. However, they agreed that the currently present techniques for purifying EVs could achieve relative EV separation (i.e. from non-vesicular co-contaminants) and/or EV concentration/enrichment (i.e. increase number of EVs per unit of volume).

There exist a large number of EV separation and concentration techniques that have been developed in the past few decades (95, 96). Ultracentrifugation (including differential ultracentrifugation) remains by far the most common technique with 80% of EV studies using it, while the remaining 20% of studies use density gradient ultracentrifugation, filtration, size-exclusion chromatography, precipitation or combined techniques. These techniques have been extensively studied and compared in terms of the resultant recovery and specificity of EV or EV subtypes, and it was noted that different methods, unpredictably, result in variable composition of EVs and subfractions of EV subgroups (97-99).

1.3.4 EVs Characterization

To characterize an EV preparation, it is recommended to analyze the isolated EVs' size distribution, concentration, protein composition and their singular vesicles (94). EV size distribution and its concentration could be analyzed using one of the following techniques:

nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), high-resolution flow cytometry or Resistive Pulse Sensing (RPS) (94). These techniques were shown to record differ rent EV size distributions for the same sample with no obvious superiority in accuracy of any of them (100). It is also why there is reluctance in nominating any of them as the gold standard method for EV quantitative analysis just yet (94).

To characterize EVs by their protein composition, quantitative and/or qualitative analyses of proteins in or on EVs could be conducted using several methods, such as mass spectrometry, western blotting or flow cytometry. ISEV's recommendations entail that several protein markers must be analyzed to demonstrate the presence of EVs and their relative purity from commonly co-isolated contaminants. They divided these protein markers into three main categories, and recommended the EV preparation in question should demonstrate at least one protein from category 1 and 2.

Category 1 is a list of transmembrane or GPI- anchored proteins associated with plasma membrane and/or endosomes, whose presence demonstrate the lipid-bilayer structure of EVs. Category 2 is a list of cytosolic or periplasmic proteins whose presence demonstrates that the EV sample examined displays the structure of lipid bilayers enclosing intracellular materials, which is also expected with all EVs. Category 3 is a list of proteins, which are constituents of common non-EV structures often co-isolated with EVs. Evaluation of these proteins helps to assess the degree of purity of EV preparation; however, there is no proposed threshold of abundance of these proteins below which acceptable purity is reached.

Besides demonstrating the general presence of EVs, these protein markers could potentially be utilized to give an idea about which EV subtypes may be present in the analyzed sample. However, they should be examined conservatively and with extreme caution since

different EV isolation approaches and different cellular sources of these EVs, yield different EV protein compositions (101-104). For this reason, it has been clearly highlighted in the latest ISEV position statement that "it is still not possible to propose specific and universal markers of one or the other types of EVs" (94).

1.3.5 Role of EVs in tissue regeneration

In the context of tissue repair, mesenchymal stem cells exert their functions, at least partially, through paracrine mechanisms by secreting packaged extracellular factors (105, 106). Subsequently, studies have shown that through this transfer of biomolecules, mesenchymal stem cell-derived EVs (MSC-EVs) could potentially hold regenerative effects in various tissues and organs. In a myocardial infarction animal model, MSC-EVs treatment following heart infarction resulted in a significant improvement in cardiac function, reduction in infarct size and prevention of heart remodeling (107, 108). Similarly in animal stroke models, MSC-EVs were able to induce neuroregeneration, improve functional recovery and provide long-term neuroprotection (109, 110). Several other studies have also reported renoprotective and hepatoprotective effects of MSC-EVs (111, 112) in respective animal kidney and liver disease models. These findings further enforced the premise of MSC-EVs' immense regenerative potential and raised the likelihood of establishing similar roles in other tissues, such as in bone.

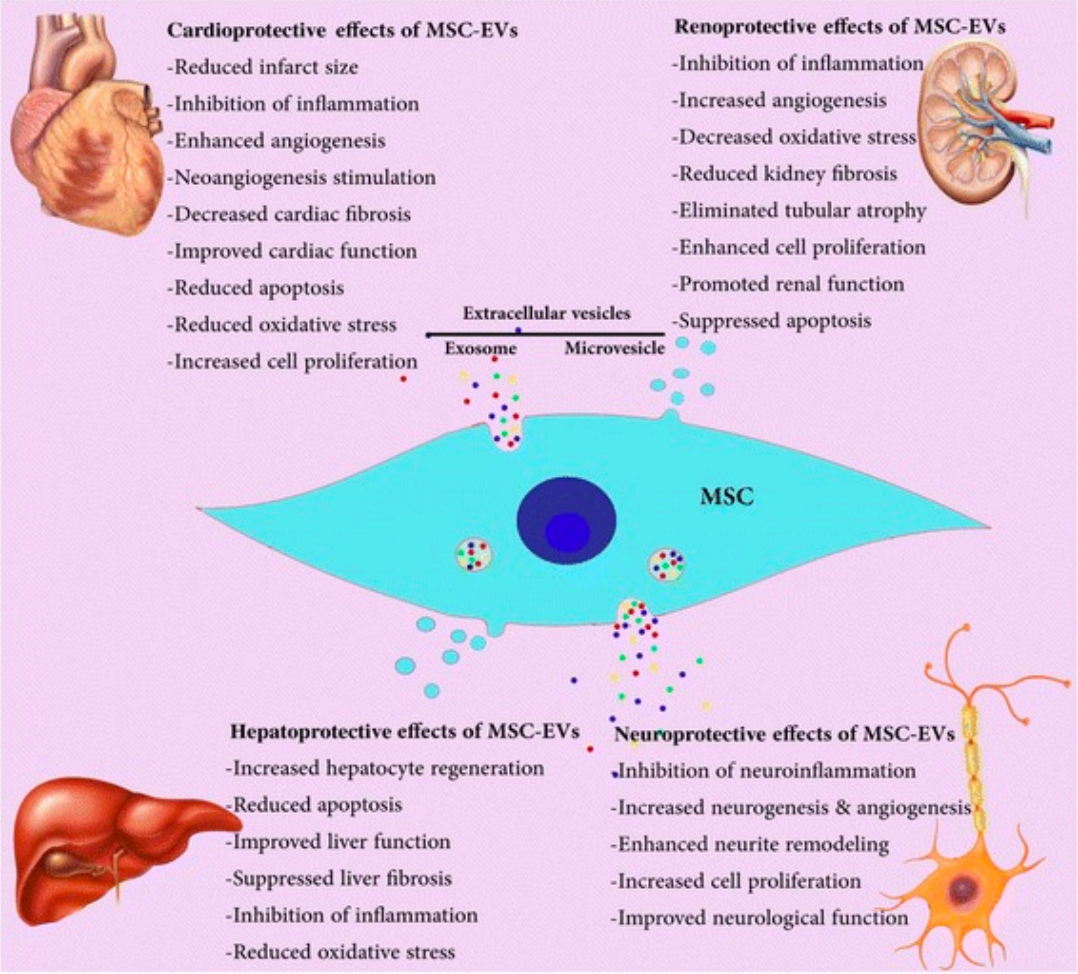

Figure 1.4: Role of EVs in tissue regeneration in various organs

Diagram demonstrating the wide-range of effects of MSC-derived EVs in various tissues, signifying regenerative and cardiac-, renal-, neural- and hepatic-protective potentials. This figure was adapted from Keshtkar et al. (113). Abbreviations- MSC: Mesenchymal Stem Cells; EV: Extracellular vesicles

Section 4: Extracellular Vesicles, bone cells and bone regeneration

1.4.1 Promotion of osteogenic differentiation

Just like MSCs, osteoblasts amongst other bone cells have been shown to interact and communicate with their microenvironment via paracrine signaling (72, 114). There is an increasing knowledge of EVs' biological role in many bone-related processes, which emphasizes the potential role they could have in bone regeneration *in-vivo*.

The key initial step of tissue regeneration is to achieve specific lineage differentiation of stem cells into more committed functional cell lines. EVs harvested from various sources have been shown to exhibit pro-osteogenic abilities by their capacity to drive stem cell differentiation into the osteoblastic lineage. For example, EVs derived from osteogenic MSCs were able to induce undifferentiated human bone marrow MSCs to differentiate *in-vivo* and *in-vitro* (115). *In-vitro*, EV-mediated differentiation was evidenced by the elevated expression of genes and proteins involved in matrix mineralization, vascularization and osteogenic differentiation, such as BMP2, TGFβ, PDGF, VEGF and tubulin. *In-vivo*, the EVs and MSCs were seeded on a collagen scaffold then implanted subcutaneously in athymic nude mice for four weeks. Histological evaluation of the implant revealed successful mineralization with Von Kossa and Alizarin Red S stains, as well as robust vascularization with Hematoxylin and Eosin (H&E) stain.

MC3T3-E1cells, a mouse calvarial pre-osteoblast cell line, were induced to differentiate to mineralizing osteoblasts, from which EVs were isolated and characterized (116). Microarray analysis of these EVs revealed content rich in microRNAs (miRNAs), which are noncoding RNAs that regulate posttranscriptional expression of target genes, some of which were miR-1192, miR-680 and miR-302a. These EVs then stimulated bone marrow stromal cells to

differentiate, as was evidenced by the up-upregulation of osteogenic gene markers and the mineralization of the extracellular matrix. They hypothesized that the EVs achieved osteogenic differentiation as a result of the miRNAs inhibiting Axin1 expression and thus activating Wnt/β-catenin signaling pathway, which is a major osteoblast differentiation/osteogenesis pathway (117).

1.4.2 Regulation of osteoblast's behavior

EVs harvested from bone marrow-MSC were characterized and their RNA content analyzed (118). These EVs were rich in microRNA-196a, which is a key factor in stimulating cell proliferation and activity and inhibiting its apoptosis (119). As such, there was a statistically significant upregulation of several osteogenic genes following osteoblast treatment with these EVs, when compared to a no EV treatment group. Marginal effect on osteoblast proliferation following EV treatment was also noted with MTT assay, which is a colorimetric assay that assesses cell metabolic activity, viability and proliferation (120).

1.4.3 EVs and the bone's internal regulatory system

Bone remodeling ensures maintenance of a healthy bone through a dynamic balance in function between the bone-forming osteoblasts and the bone-resorbing osteoclasts (1). For this reason, bone researchers were interested in exploring the possible role of EVs in regulating the function of bone cells involved in bone remodeling.

Multiple myeloma cell-derived EVs were able to induce a macrophage cell line (osteoclast precursors) to mature and differentiate into multinuclear osteoclasts (121). This was shown by a significant elevation in the expression of osteoclast gene markers, such as Cathepsin

K, Metalloproteinases 9 and Tartrate-Resistant Acid Phosphatase (TRAP). The multinucleated osteoclasts were then grown on dentine (bone) discs and further treated with these EVs. This resulted in the formation of lacunae (cavities) on the discs, thereby demonstrating the ability of EVs to stimulate osteoclast bone resorption activity. Another group analyzed the content of rat osteoblast- and osteocyte-EVs (122), which revealed the presence of RANK ligand and OPG that are critical for regulating osteoclast differentiation (49, 123, 124). Interestingly also, several studies have analyzed the content of osteoclast-derived EVs (125-128), which revealed the presence of numerous bone-regulatory proteins and miRNAs that are involved in modulating osteoblastic bone formation.

Altogether, these findings suggest the presence of constant intercellular communication between osteoblasts and osteoclasts, through EV secretion, to regulate bone formation and resorption.

1.4.4 EVs and *in-vivo* bone regeneration

MSC-EVs have been shown to possess *in-vivo* bone formation/healing capabilities in several animal models. One study reported that human embryonic MSC-derived EVs induce osteochondral regeneration in a rat model of critical-sized calvarial defect (129). Similarly, bone marrow MSC-derived EVs were capable of promoting femoral fracture healing in CD9-null mice (a strain that is known to produce reduced levels of EVs) (130). This demonstration of powerful bone regeneration properties could make EVs a future tool for treating bone defects and enhancing bone growth.

Section 5: Study Objectives and Hypotheses

Despite numerous studies investigating OI, there is still plenty of deficiencies in our understanding of this disorder and its complex pathogenesis, particularly at a cellular level. This contributes to the fact that to this day, there is no known treatment, medication or surgery that cures OI or effectively prevents its complications.

Hence, in this research project, we want to expand our knowledge of the OI-cells and their paracrine behavior, which was never studied before and remain to be elucidated. Given OI is often caused by mutations in *COL1A1* genes, we will be conducting our experiments using cells derived from OI patients with this genotype.

Our objectives and aims are to:

1. Optimize a protocol for enriching and concentrating EVs from human-derived MSCs and their derivative Obs (this is not a hypothesis-driven aim).
2. Isolate EVs from *COL1A1* OI- and human control-MSCs and their derivative Obs. We hypothesize that we will be able to adequately enrich and characterize said EVs.
3. Analyze and compare the OI- and human control-EVs, with specific focus on their proteomic content. We hypothesize that there will be proteomic differences between the OI- and human control-Obs-EVs, which would highlight any deviation of OI-Ob behavior and shed some light on OI pathogenesis.

Chapter 2: Materials and Methods

2.1 Overview

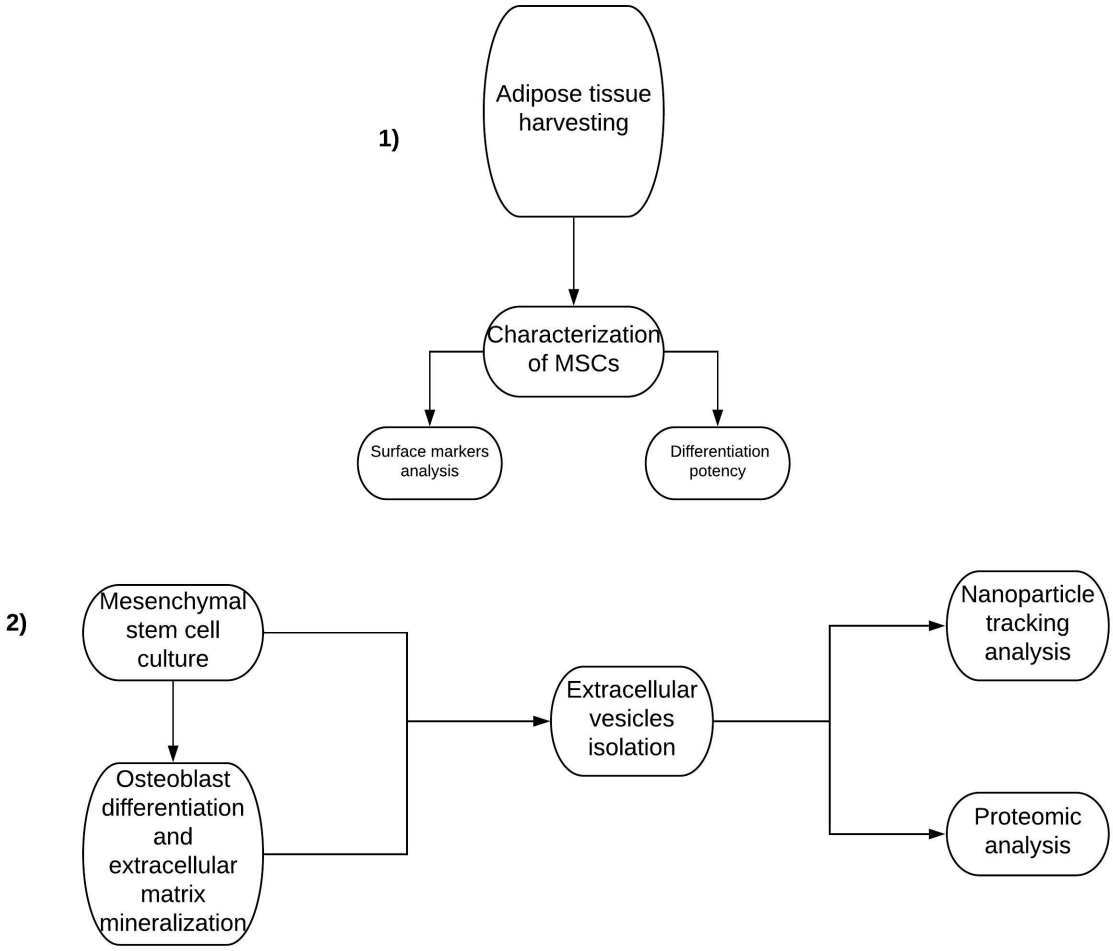

Figure 2.1: Overview of the methods of the study

A schematic flowchart illustrating an overview of the two main steps of the study methods

1. Isolation of adipose tissue-derived MSCs and their characterization.
2. Isolating EVs from tissue culture, and subsequent methods of analysis.

Abbreviations- MSCs: Mesenchymal stem cells, EVs: Extracellular vesicles

2.2 Adipose Tissue harvesting

Our source of Mesenchymal stem cells (MSCs) was from the subcutaneous adipose tissue of two OI patients with *COL1A1* mutations and one human control patient (i.e. not diagnosed with OI or any other bone metabolic disease). These patients were admitted under the pediatric orthopedic surgery department and scheduled to have elective surgery for correction of a bone deformity. In the human control patients, these deformities were physiological and not due to any underlying disease. One fat sample was harvested from the surgical site of each of the three patients. The below table briefly demonstrates the demographics of the patients (Table 2.1). As will be detailed below, MSCs harvested from each of these fat samples were cultured in duplicates to give two samples for each and yield a total of six MSC groups (four OI-MSCs and two HC-MSCs).

Diagnosis/ type of OI	Gene mutated	Nucleotide change	Amino acid change	Age (yrs)	Sex
HC	None	None	None	10	F
OI-IV	COL1A1	c.4325_4335delTGGCCCCCTTG	p.Val1442Glyfs*105	6	F
OI-IV	COL1A1	C.2461G>A	P.GLY821SER	5	F

Table 2.1: Demographics of the study subjects

The MSCs of our study were obtained from the adipose tissue of three patients. This table illustrates the demographics of these patients: diagnosis/ type of OI, mutated gene, nucleotide change, age, sex and the number of adipose tissue samples harvested. Abbreviations- HC: Human control; OI: Osteogenesis Imperfecta; COL1A1: α1- chain type-I collagen

2.3 Isolation of MSCs

Following dissection of the fat sample during the surgery, it was transferred in a sterile container to our laboratory in less than 10 minutes for processing. Then using a protocol developed in our lab, MSCs were isolated from the patient's adipose tissue. To start with, the fat sample was cut into small pieces using surgical scissors to allow adequate digestion by a solution of 2.2mg/ml collagenase enzyme (clostridium histolyticum Type IA collagenase, SIGMA C-9891), Bovine Serum Albumin (0.3 g of BSA/g of fat, SIGMA A7030-50G) and Hanks balanced salt solution (HBSS) (SIGMA H6648). The digested mixture was filtered using 0.22μm Millipore filters, then centrifuged at 700xg for 10 minutes and discarded the supernatant. A pellet containing MSCs and other cell types was washed and then suspended in complete growth medium: Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (DMEM:F12, Gibco, Grand island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Grand Island, NY, USA), 1% antibiotic-antimycotic (A/A, Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin (P/S, Gibco, Grand Island, NY, USA).

2.4 MSC culture expansion and characterization

We then seeded the cells in 25cm^2 tissue culture flask and changed the media the next day to remove non-adherent cells, as mesenchymal stem cells adhere to the flask/plastic (17). Cell proliferation was stimulated by replacing the media on the flask every three days with complete growth media. This was continued until cell confluency of 70-80% was achieved, after which the cells were trypsinized TrypLE Enzyme (Gibco, Grand Island, NY, USA) and reseeded in a 75cm^2 tissue culture flask at a cell density of 3000cells/cm^2.

To characterize/confirm the identity of the cultured cells to be MSCs, their surface markers and differentiation potency were assessed. The MSCs were stained for the following

fluorochrome-conjugated monoclonal antibodies (BD Pharmingen Inc, San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated anti-CD90, anti-CD45, phycoerythrin (PE)-conjugated anti-CD73 and allophycocyanin (APC)-conjugated anti-CD34. Data analysis was then performed using FlowJo software. To assess for MSC's osteogenic potency, the cells were seeded at a cell density of 3000cells/cm^2 in a 6 well plate and were induced to differentiate into osteogenesis. Osteogenic treatment consisted of complete growth media with 10nm dexamethasone (Sigma, Darmstadt, Germany), 10mM β-glycerophosphate (Sigma) and 50µg/ml Ascorbic-Acid-2-phosphate (Sigma). Treatment medium was replaced every three days, and on day21 following treatment initiation, Alizarin Red S Stain (Sigma) was used for histological evaluation of osteogenesis. Because of our interest in MSCs and their osteogenic lineages only, we did not assess the MSCs capacity to differentiate into other lineages.

Each type of MSCs derived from the three different fat samples was cultured in duplicate. This resulted in a total of six MSC cultures that will be from here onward treated as separate samples (a total of four OI-MSCs and two HC-MSCs).

2.5 Fetal Bovine Serum EV-depletion

Prior to using FBS as a supplement for the culture growth medium in our experiments, EVs and other contaminants were first depleted. As well highlighted in the latest ISEV position statement, absolute depletion/isolation of EVs is not achievable by any technique (94). We aim; however, to achieve relative depletion of EVs from these contaminating sources such that our analysis of the EVs isolated from our culture cells won't be significantly affected.

As summarized in Figure 2.2 below, we did so by a combination technique of serial differential ultracentrifugation and ultrafiltration of the FBS, to yield an FBS supernatant depleted of EVs. First, we centrifuged the FBS at 2000xg at room temperature for 10 minutes

then filtered the supernatant by pouring it into a vacuum-connected 0.22µm filter on top of a sterile bottle. The filtrate was centrifuged at 10,000xg at room temperature for 30 minutes using an SW 32 Ti rotor (Beckman Coulter). The pellet was then discarded and supernatant centrifuged at 100,000xg at 4°C for 70 minutes. The supernatant of this last centrifuge is the EV-depleted FBS that we used to supplement our growth medium.

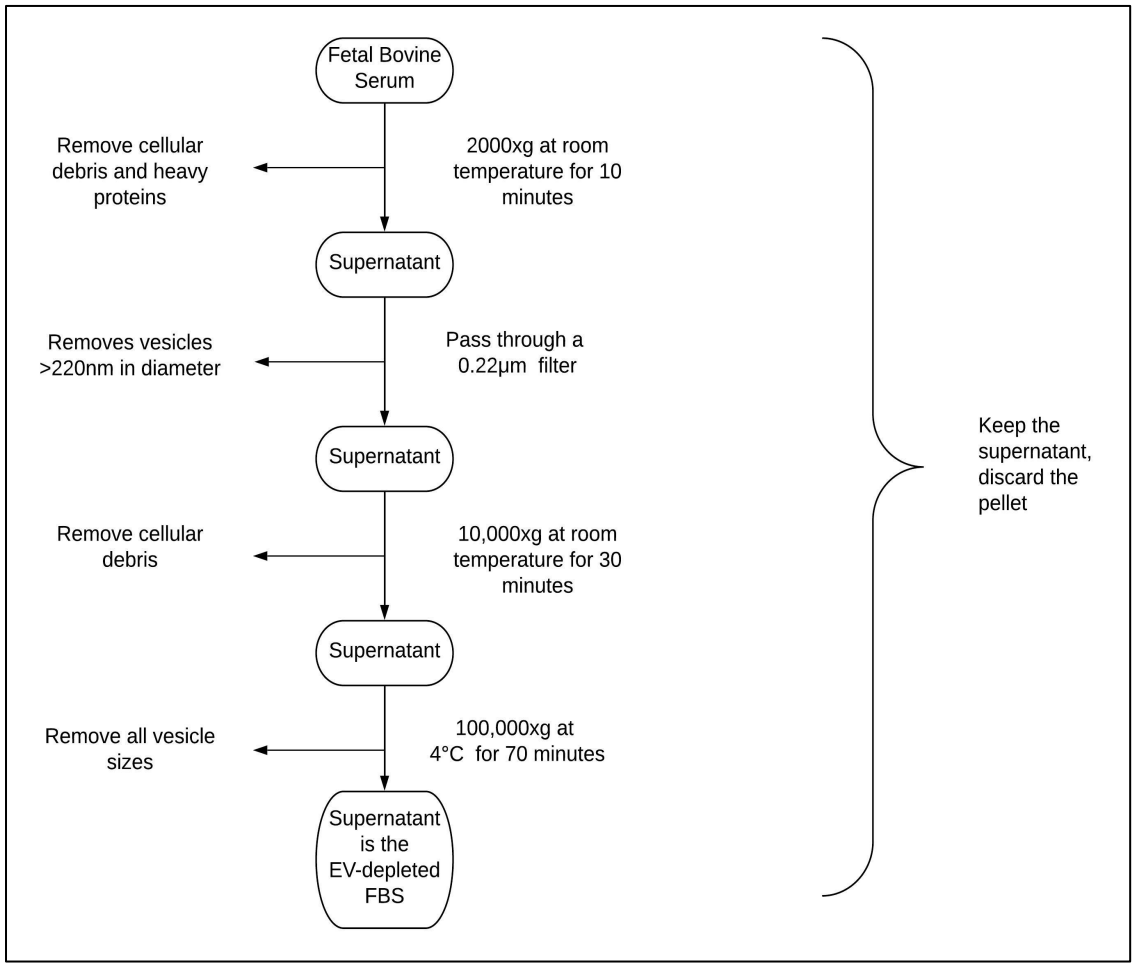

Figure 2.2: Method of depletion of EVs and other contaminants from FBS

Schematic flowchart demonstrating the steps of the combined ultrafiltration and differential ultracentrifugation technique used to deplete EVs and other contaminants from FBS. EV-depleted FBS was used to supplement the cell culture media of the cells whose secreted EVs were to be studied. Abbreviations- EVs: Extracellular Vesicles; FBS: Fetal Bovine Serum

2.6 Cell culture supernatant collection

The starting point cell types were four osteogenesis imperfecta-derived MSCs (OI-MSCs) and two human control-MSCs (HC-MSCs), at passages 3-5. The MSCs were seeded at a cell density of 3000cells/cm^2 in two 175cm^2 tissue culture flasks under standard conditions (5% carbon dioxide, 37°C). They were cultured in complete growth media using 10% EV-depleted FBS and were left to proliferate until ≈ 70-80% confluency was achieved. Throughout this period, which took 7-10 days, media was collected every three days from both flasks, and all of these collections were pooled together to form what we call the 'non-treatment' group. After achieving ≈ 70-80% confluency, osteogenic treatment was initiated (Day0) using EV-depleted complete growth media, 10nm dexamethasone, 10mM β-glycerophosphate and 50µg/ml Ascorbic-Acid-2-phosphate. Osteogenic treatment was maintained for 21 days with the same constituents as the induction/initiation osteogenic treatment, with just a reduction in the working concentration of β-glycerophosphate to 5mM from day3 onward. We know from our expertise in MSCs, Obs and extracellular matrix mineralization that it takes 14 days (Day0 to 14 of osteogenic treatment) for MSCs to differentiate to Obs, and another 7 days (Day14 to 21) for the osteoblasts to fully mineralize the extracellular matrix. Thus, from both flasks of each sample/cell type, we collected culture medium every three days from days 14 post-treatment initiation to day21 (experimental endpoint). These cell culture supernatant collections were pooled together to form what we call the 'Treatment' group. As a result, we had a total volume of 100-120ml of culture medium supernatant that was collected during the non-treatment and treatment phases of the cell culture of each cell type.

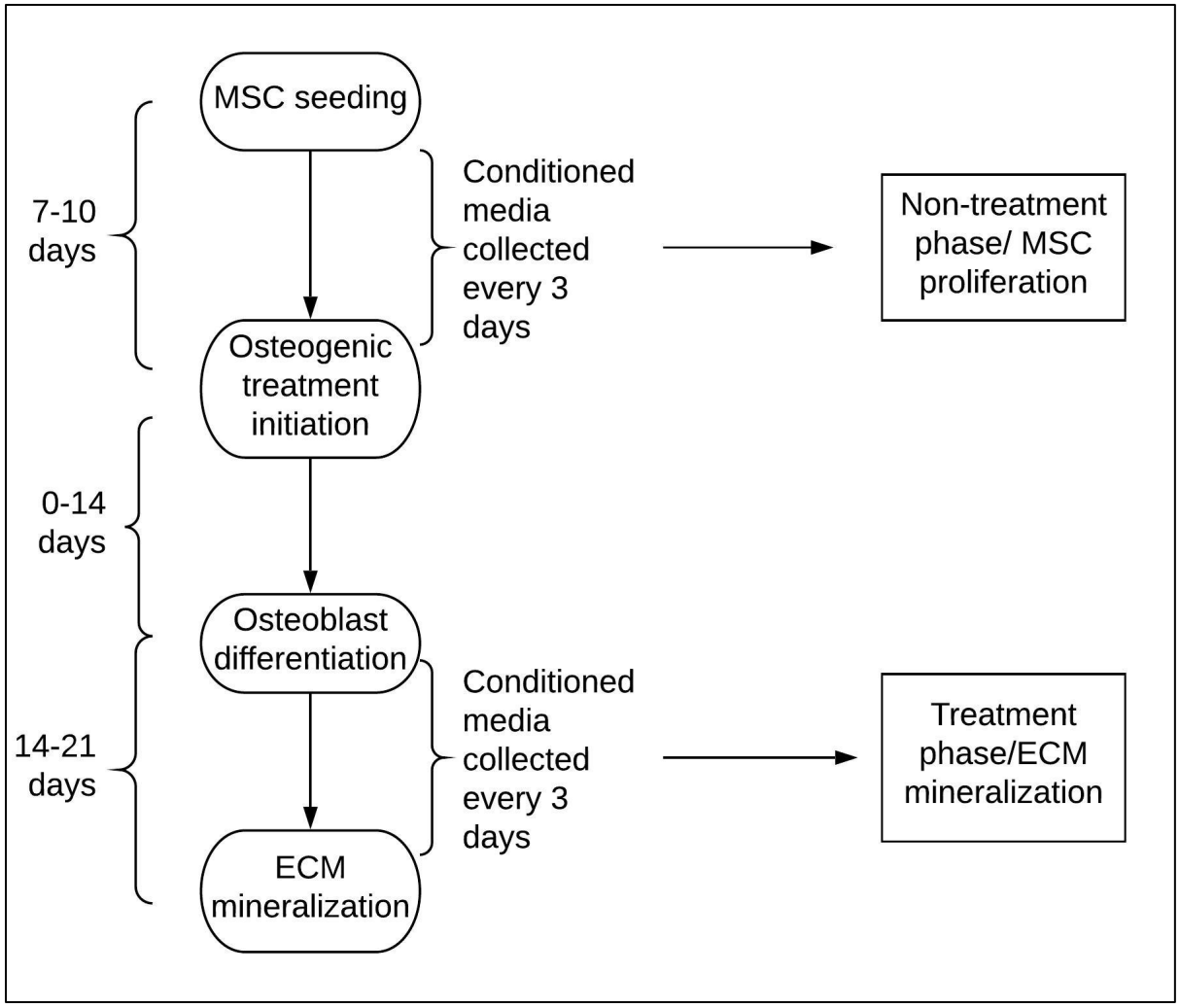

Figure 2.3: Experimental design- MSC proliferation, Ob differentiation and ECM mineralization

OI- and HC-MSCs were seeded and proliferated for 7-10 days during which cell culture supernatant was collected and pooled. After that, MSCs were induced to differentiate into osteoblasts and during the ECM mineralization phase, culture supernatant was collected and pooled. As a result, we obtained two collections of culture supernatant from each sample: one during the non-treatment phase and the other during the treatment phase.

Abbreviations- OI: Osteogenesis Imperfecta; HC: Human Control; MSCs: Mesenchymal stem cells; Ob: Osteoblasts; ECM: Extracellular matrix

2.7 Matrix mineralization histological evaluation

At day21 of osteogenic treatment, all flasks were assessed for extracellular matrix (ECM) mineralization. Histological evaluation was done using Alizarin Red Stain to visualize calcium deposits. The cells were fixed with 70% Ethanol at -20°C for 1 hour then stained with 40 mM (pH 4.2) Alizarin Red S (ARS) for 10 minutes at room temperature. The flasks were then washed with distilled water and Phosphate-Buffered Saline (PBS, pH 7.4, Gibco Grand Island, NY, USA) to remove the excess stain. Mineral/calcium deposition appears as red with the ARS stain.

2.8 EVs enrichment and concentration

As detailed previously, from each sample we obtained two pools of conditioned media collections: a non-treatment and a treatment group. To enrich and concentrate EVs from each of these cell culture supernatants, we developed a modified version of a protocol published by Thery et al in 2006 (95) (Figure 2.4). The pooled conditioned medium culture supernatant was centrifuged at 2000xg room temperature. The pellet containing dead cells was discarded, and the supernatant was then filtered by pouring it into a vacuum-connected 0.22μm filter on top of a sterile bottle. Using an SW 32 Ti rotor, the filtrate was then centrifuged at 10,000xg at room temperature for 30 minutes to remove cellular debris, which is found in the resultant pellet. The supernatant was then centrifuged at 100,000xg at 4°C for 70 minutes. The resultant supernatant was discarded, and the pellet containing EVs and contaminating proteins was washed and suspended with PBS. The last centrifuge was again at 100,000xg at 4°C for 70 minutes; the resultant supernatant containing contaminating proteins was discarded and the pellet containing the EVs was resuspended in 100-200 μl of PBS. As a result, we ended up with 12 groups of EV preparations: four OI non-treatment EVs, two HC non-treatment EVs, four OI-treatment EVs and two HC-treatment EVs.

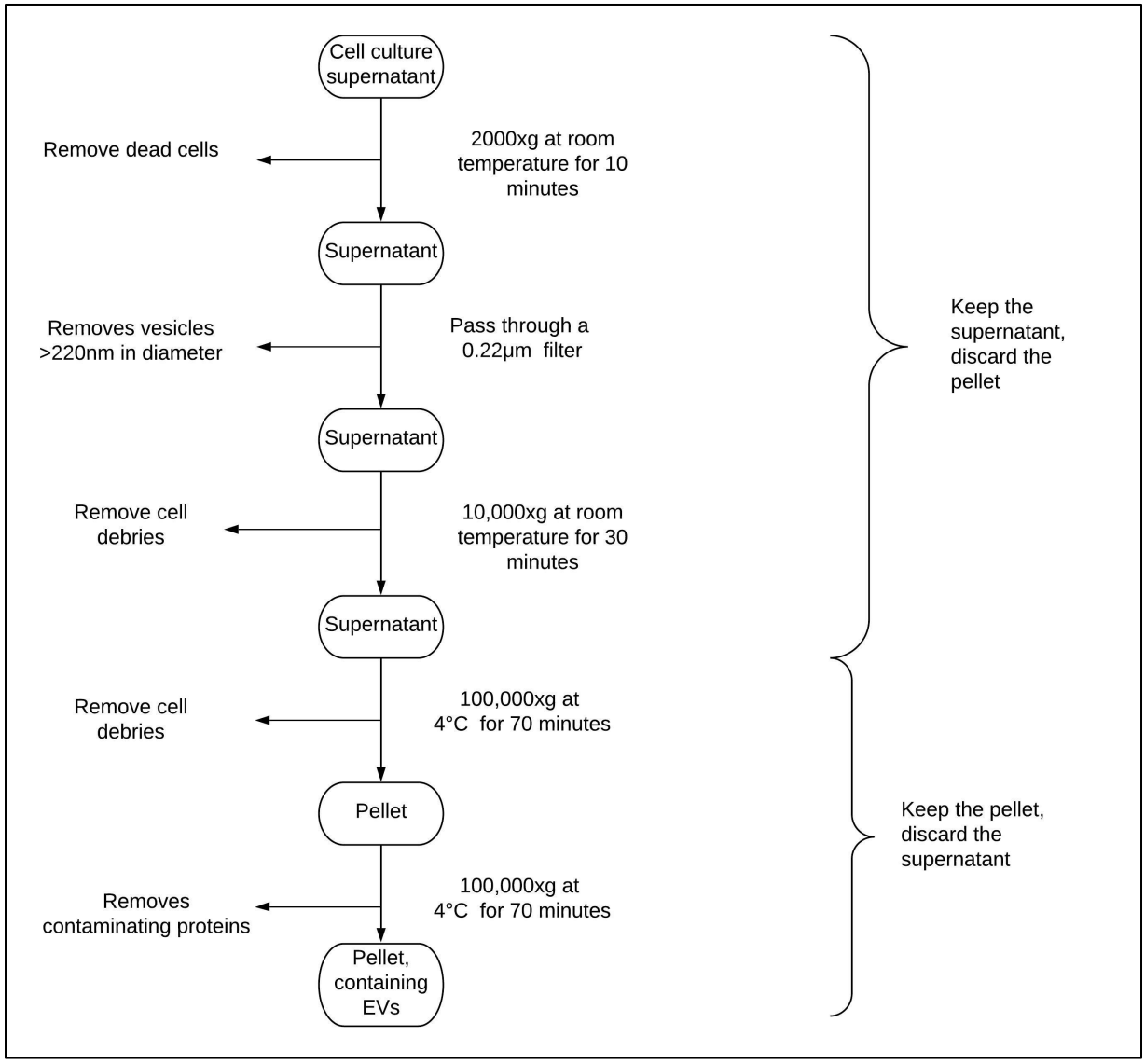

Figure 2.4: Enriching and concentrating EVs from the cell culture supernatant

Using a combination technique of ultrafiltration and serial differential ultracentrifugation, EVs were enriched and concentrated from the cell culture supernatants collections of our OI- and HC-cells. Dead cells, cellular debris, large vesicles and contaminating proteins present in the cell culture supernatants were discarded. Abbreviations- EVs: Extracellular vesicles; OI: Osteogenesis Imperfecta; HC: Human Control.

2.9 EVs Nanoparticle tracking analysis

After passing each conditioned medium collection through the 0.22μm filter, 1ml of the filtrate was obtained to analyze EV concentration and particle size distribution by NanoSightNS500 instrument and nanoparticle tracking analysis (NTA) software (Malvern, Worcestershire, UK) (131). NTA is a method that visualizes nanoparticles in a liquid by illumination with a laser beam, and captures five-30 second videos at room temperature of them. It then tracks the Brownian motion of the individual vesicles to calculate their size distribution and concentration (131).

2.10 Liquid chromatography tandem-mass spectrometry

Tryptic peptide analyses by nanospray liquid chromatography tandem-mass spectrometry (nanoLC MS/MS) was performed by the Proteomics Platform of the Research Institute of the McGill University Health Center (RI-MUHC). 1 μg of proteins (per EV preparation) were loaded onto a single stacking gel band to remove lipids, detergents and salts. The gel band was reduced with DTT, alkylated with iodoacetic acid and digested with trypsin. Extracted peptides were re-solubilized in 0.1% aqueous formic acid and loaded onto a Thermo Acclaim Pepmap (Thermo, 75uM ID X 2cm C18 3uM beads) precolumn and then onto an Acclaim Pepmap Easyspray (Thermo, 75uM X 15cm with 2uM C18 beads) analytical column separation using a Dionex Ultimate 3000 uHPLC at 220 nl/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over two hours. Peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer operating at 120,000 resolution (FWHM in MS1) with HCD sequencing (15,000 resolution) at top speed for all peptides with a charge of 2+ or greater. The raw data were converted into *.mgf format (Mascot generic format) for searching using the Mascot 2.5.1 search

engine (Matrix Science) against human protein sequences (Uniprot 2018). The database search results were loaded onto Scaffold Q+ Scaffold_4.4.8 (Proteome Sciences) for statistical treatment and data visualization.

Chapter 3: Results

3.1 MSCs fullfil the ISCT criteria

The OI- and HC-MSCs derived from the six human adipose tissue samples met the minimal criteria set by the ISCT for defining MSCs (16). They all had a spindle-shaped morphology and plastic-adherence properties as seen in Figure 3.1A. Both types of MSCs were also capable of osteogenic differentiation and ECM mineralization, as evident by the bright red calcium deposits seen by histological evaluation with Alizarin Red S staining (Figure 3.1B). This finding supports the idea that the isolated cells are stem cells as opposed to mesenchymal stromal cells. They also met the minimum immunophenotypic requirement of expressing surface markers CD90, CD34 and CD73, and lacked the expression of CD45 (Figure 3.1C).

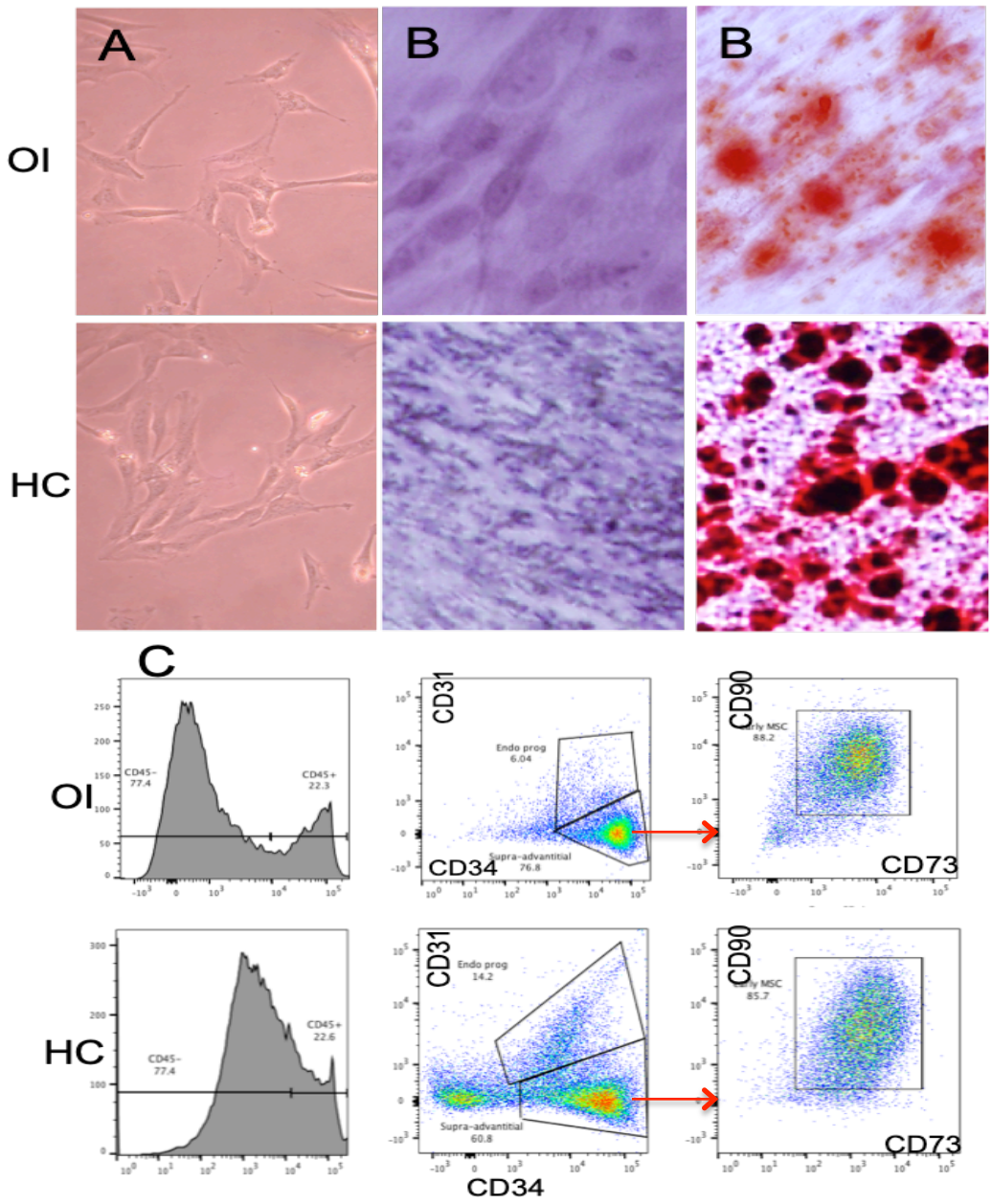

Figure 3.1: Characterization of adipose tissue-derived MSCs

Figure illustrating the characterization of our OI- and HC-MSCs, where (A) shows their spindle-shaped morphology & plastic adherence property, (B) shows their osteogenic differentiation by ARS stain (right) with undifferentiated MSCs used as a control (left), and (C) shows they are positive for CD34, CD73 & CD90, and negative for CD45. Abbreviations –OI: Osteogenesis Imperfecta; HC: Human control; MSC: Mesenchymal stem cells; ARS: Alizarin Red S

3.2 EVs isolation from proliferating MSCs and mineralizing Obs

We were able to successfully induce human control- and Osteogenesis Imperfecta-derived Mesenchymal stem cells to differentiate into osteoblasts that resulted in extracellular matrix mineralization. This mineralization was confirmed histologically with Alizarin Red S (ARS) stain evaluating the presence of calcium deposits, which appear as bright red. As a reference point, ARS stain was also done prior to initiating osteogenic treatment (i.e. of the MSCs culture) and, as expected, showed no calcium deposition (Figure 3.2).

During the non-treatment period, conditioned culture medium was collected, which contained EVs that were secreted by MSCs during their proliferation. During the treatment period, day14 to 21, culture medium collections contained EVs secreted by osteoblasts during the extracellular matrix mineralization stage. Hence, EVs isolated from the non-treatment group/proliferating MSCs and the treatment group/ECM mineralizing-osteoblasts will be referred to from here onwards as MSCs-EVs and Ob-EVs respectively. Thus in total, we had four OI-MSC-EVs, two HC-MSC-EVs, four OI-Ob-EVs groups and two HC-Ob-EVs samples.

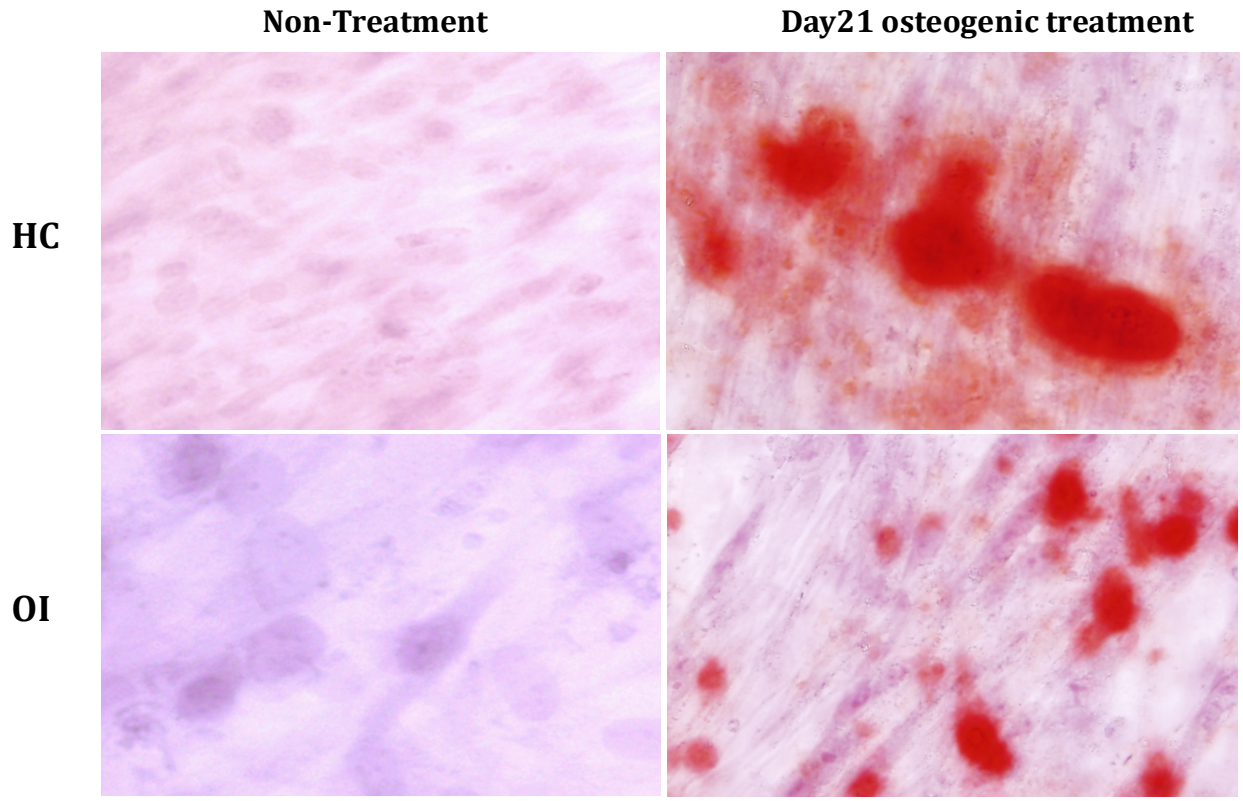

Figure 3.2: ARS stain for histological evaluation of matrix mineralization

ARS stain was used for histological evaluation of ECM mineralization by assessing for the presence of calcium deposits (appear as bright red). This figure shows stained cell cultures of undifferentiated cells (MSCs) and differentiated/mineralized cells (Obs): HC-MSCs (top left), OI-MSCs (bottom left), HC-Obs (top right) and OI-Obs (bottom right). Abbreviations- ECM: extracellular matrix; HC: Human control; OI: Osteogenesis Imperfecta; MSC: mesenchymal stem cell; Ob: Osteoblast; ARS: Alizarin Red S

3.3 EVs size distribution and concentration analysis

We quantitatively assessed our EV preparations as per the latest International Society of Extracellular Vesicles position paper's recommendation for EV characterization (94). We performed Nanoparticle Tracking Analysis for the MSC- and Ob- EV preparations of the HC and OI cells. The results were displayed on a histogram representation of the EV size distribution and EV average size, mode particle size, size range and concentration (number of particles/ml) were calculated.

For all the EV preparations, the histogram representation of the EV size distribution had a bell-shaped curve recording mode particle size (the particle size most commonly observed) between 150 to 250nm (Figure 3.3A). The average sizes of the EVs of the OI- and HC-MSCs were approximately 250 and 244nm respectively, and those of the OI-Obs and HC-Obs were approximately 262 and 248nm respectively (Figure 3.3B). In addition, there were no statistically significant differences between the average sizes of OI- and HC-EVs (i.e. p-value>0.05).

The average concentration of EVs in 1ml (number of vesicles/ml) of collection media secreted by the cells was also analyzed by NTA. The average concentration of EVs by the OI- and HC-MSCs were 1.25E+09 and 1.60E+09, and by the OI- and HC-Obs were 2.10E+09 and 1.84E+09 respectively (Figure 3.3B). The average concentration of the EVs secreted by the OI-Obs was significantly more than that of OI-MSCs, p-value ≤ 0.05.

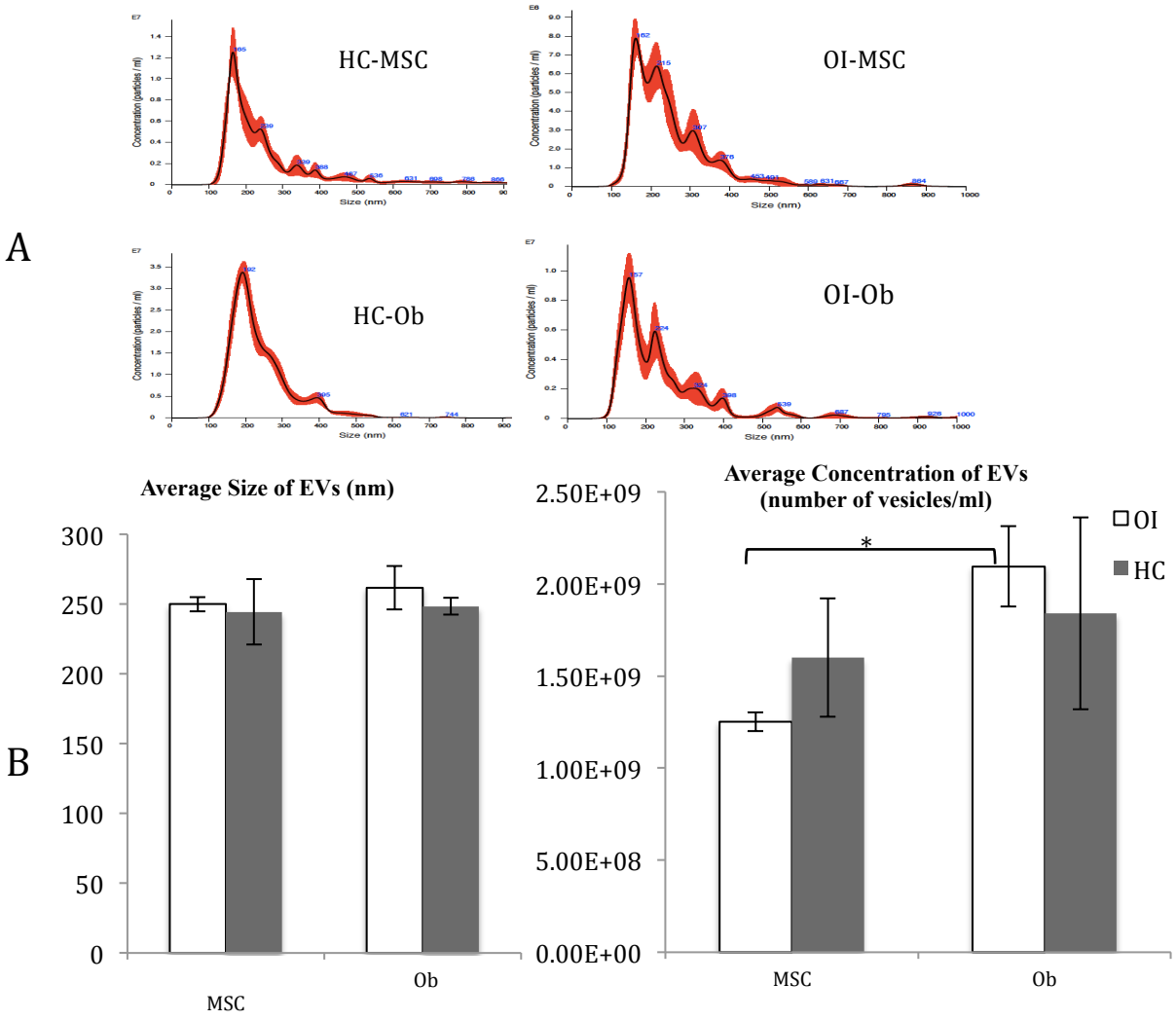

Figure 3.3: Nanoparticle tracking analysis of the EVs

A) Four histograms illustrating the EVs' size distribution and their mode particle size of a: HC-MSCs (top left), HC-Obs (bottom left), OI-MSCs (top right) and OI-Obs (bottom right).

B) Two charts illustrating the average size of the EVs (left) and average concentration of the EVs (right) of the four different EV groups: OI-MSC (n=4), HC-MSC (n=2), OI-Ob (n=4) and HC-Ob (n=2). Mean ± standard deviation is presented, where (*) represents a *p*-value ≤0.05. Abbreviations- MSC: Mesenchymal stem cells; Ob: Osteoblasts; OI: Osteogenesis Imperfecta; HC: Human control; EV: Extracellular vesicles

3.4 Proteomic analysis of isolated EVs

We subjected our proteomic results to stringent inclusion criteria of only including in our analysis proteins with a minimum peptide count of 5 in at least one sample, as well as a protein threshold of 99%. As a result, a total of 384 different proteins were identified in all of the EV groups combined, with a False Discovery Rate (FDR) of 0%. We then compared our proteomics data with Vesiclepedia, which is a published extracellular vesicles database of molecular data identified by researchers studying various classes of EVs from different cell types (132). It showed that 373 proteins (≈97% of all 384 proteins identified) were found in the Vesiclepedia database (Figure 3.4).

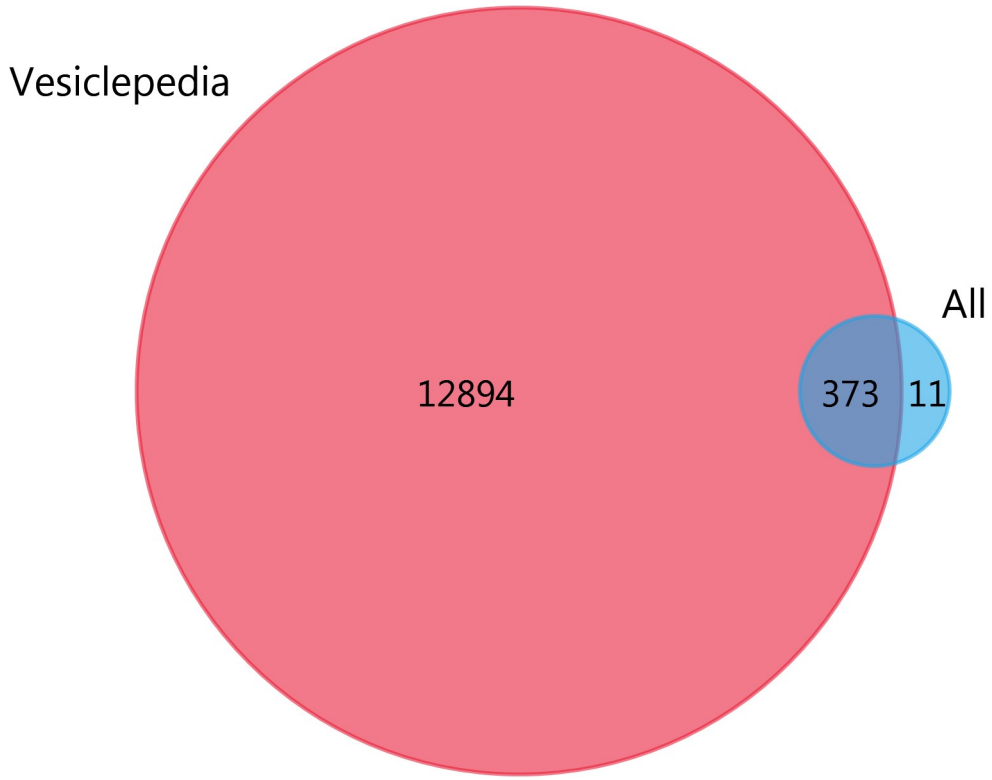

Figure 3.4: EV proteins found in Vesiclepedia

Venn diagram showing that 373 of the 384 total proteins identified in all of our EV samples combined were found in the Vesiclepedia database. The remaining 11 proteins are likely to be unique to our samples and/or never before encountered in EVs. Abbreviations- EV: Extracellular Vesicle.

The 384 proteins identified in the EV samples were then classified based by their cellular component Gene Ontology (GO) on FunRich software. It is an open software that uses customized Perl scripts to analyze functional and clinical data of proteins compiled in several databases using the human FunRich database as a background (updated database, January 2019) (133). In order to show the highest diversity of enriched GO terms, we removed duplicate or highly similar terms and selected those with the highest statistical power. The cellular

component/origin of the 384 proteins was very diverse, with the vast majority originating from extracellular exosomes (72.6%) and other cellular origins including plasma membrane (49.3%), other membranes (30.1%) and cytosol (49.3%) (Figure 3.5). The cumulative percentages may imply that some proteins may be coming from more than one cellular component or origin.

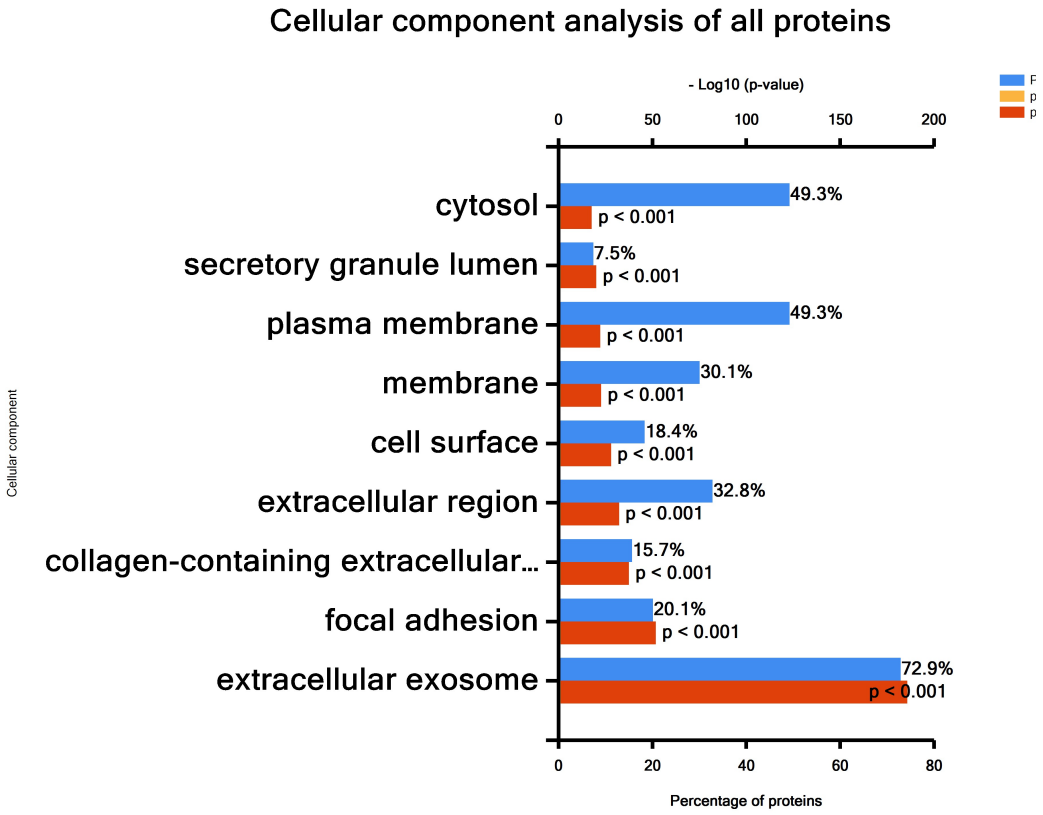

Figure 3.5: Cellular component GO enrichment analysis of all the EV proteins

Using FunRich software, cellular component GO enrichment analysis was done for the 384 proteins identified in the EV samples and displayed on a bar chart. Only the statistically significant cellular component categories were demonstrated, which showed that the detected proteins had a wide range of cellular origins, with most coming from extracellular exosomes. Abbreviations- GO: Gene Ontology

3.5 OI- and HC-Obs differentiation

To further verify that osteoblast differentiation of our cells took place, we analyzed and compared the alkaline phosphatase expression between the OI- and HC-Obs-EVs and their respective MSC-EVs. We found an exclusive expression of alkaline phosphatase in the OI- and HC-Ob-EVs relative to their MSCs.

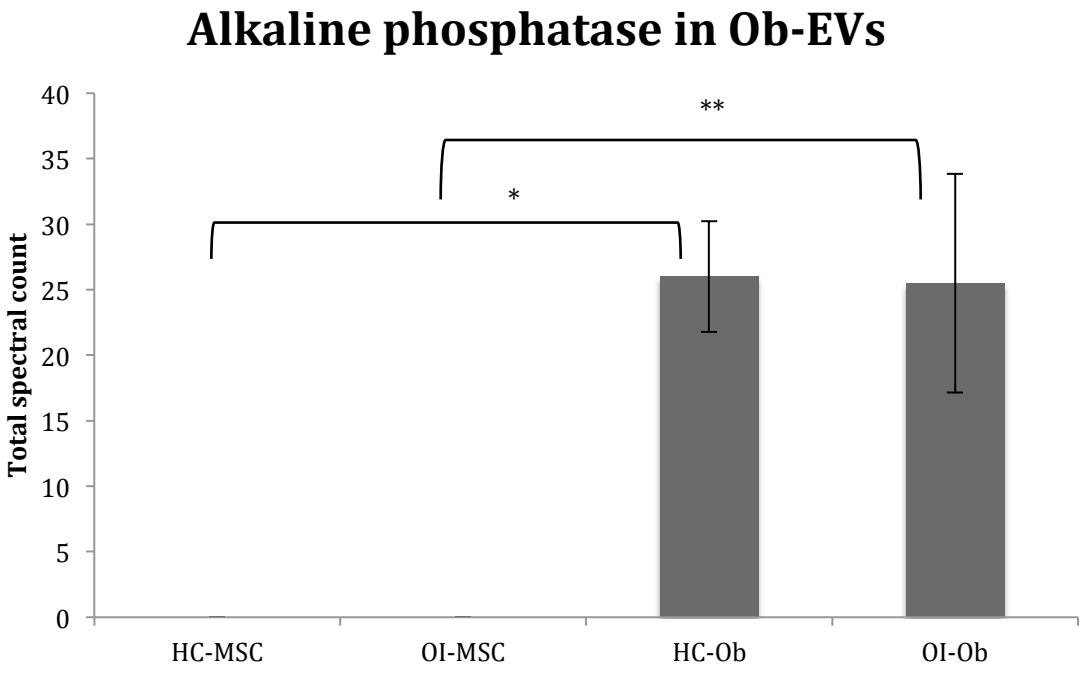

Figure 3.6: Exclusive expression of ALP by the HC- and OI-Obs

For all four OI- and two HC-MSC and -Ob groups, proteomic analysis of their EVs revealed an exclusive expression of alkaline phosphatase by all the osteoblasts and complete absence from the MSC-EVs. The figure demonstrates the average/mean total spectral count of ALP in each EV group ± standard deviation, where (**) represents a p-value$\leq$ 0.01 and (*) a p-value $\leq$0.05. Abbreviations- HC: Human Control; OI: Osteogenesis Imperfecta; EV: Extracellular vesicles; MSC: Mesenchymal stem cells; Ob: Osteoblasts; ALP: alkaline phosphatase.

3.6 Proteomic characterization of the EVs

As per ISEV's guideline for characterizing EVs using protein markers, all OI- and HC-EV groups contained at least one protein from category 1 and category 2 lists of proteins. These are transmembrane or GPI-anchored proteins and cytosolic or periplasmic proteins whose presence; thereby, demonstrates the presence of EVs in our preparations. In addition, all of our EV preparation groups contained albumin (a category 3 protein) in significant amounts. We attributed this finding to the fact that we used 10% FBS in our growth and osteogenic culture mediums.

The following category 1 and 2 proteins were found in all EV groups: Tubulin α1b (TBA1B), Tubulin β4b (TBB4B), Heat Shock Protein A8/70 (HSPA8/HSC70), Heat Shock Protein 90 β/84 (HSP90 β/84), Heat Shock Protein 70/A1A (HSP 70/A1A), Actin cytoplasmic 2 (ACTG1), Annexin A2 and Annexin A5 (Figure 3.7).

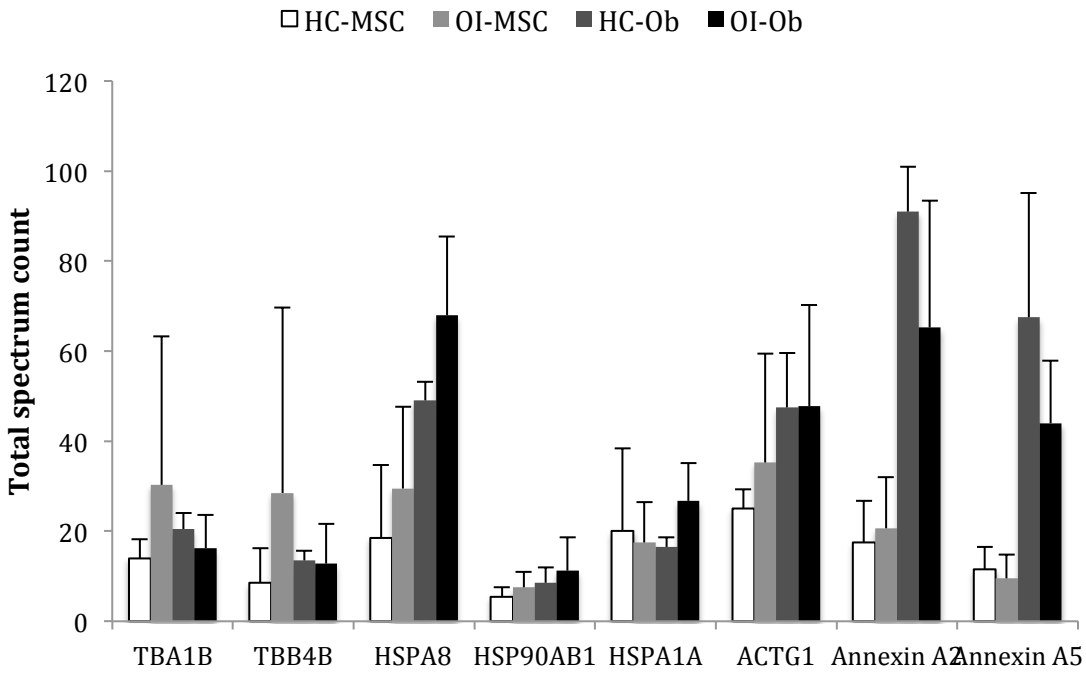

Figure 3.7: EV proteins markers found in all EV groups

All EVs of the HC- and OI-MSCs and –Obs had eight EV protein markers in common (i.e. present in all groups). This chart demonstrates the mean expression (total spectral count) of these eight proteins in each EV group ± standard deviation. Abbreviations- TBA1B: Tubulin α1b; TBB4B: Tubulin β4b; HSPA8: Heat Shock Protein A8; HSP90β: Heat Shock Protein 90β; HSPA1A: Heat Shock Protein A1A; ACTG1: Actin cytoplasmic 2.

3.7 MSC- and Ob-EVs protein marker analysis

We further analyzed the protein content of the EV preparations from the point of view of identifying which category 1 and 2 proteins are significantly upregulated in the six Ob-EVs relative to the six MSC-EVs groups. For this analysis, we conducted a t-test between the above-mentioned two groups, which identified 34 different proteins that were differentially and significantly upregulated in the Ob-EVs relative to the MSC-EVs (Figure 3.8).

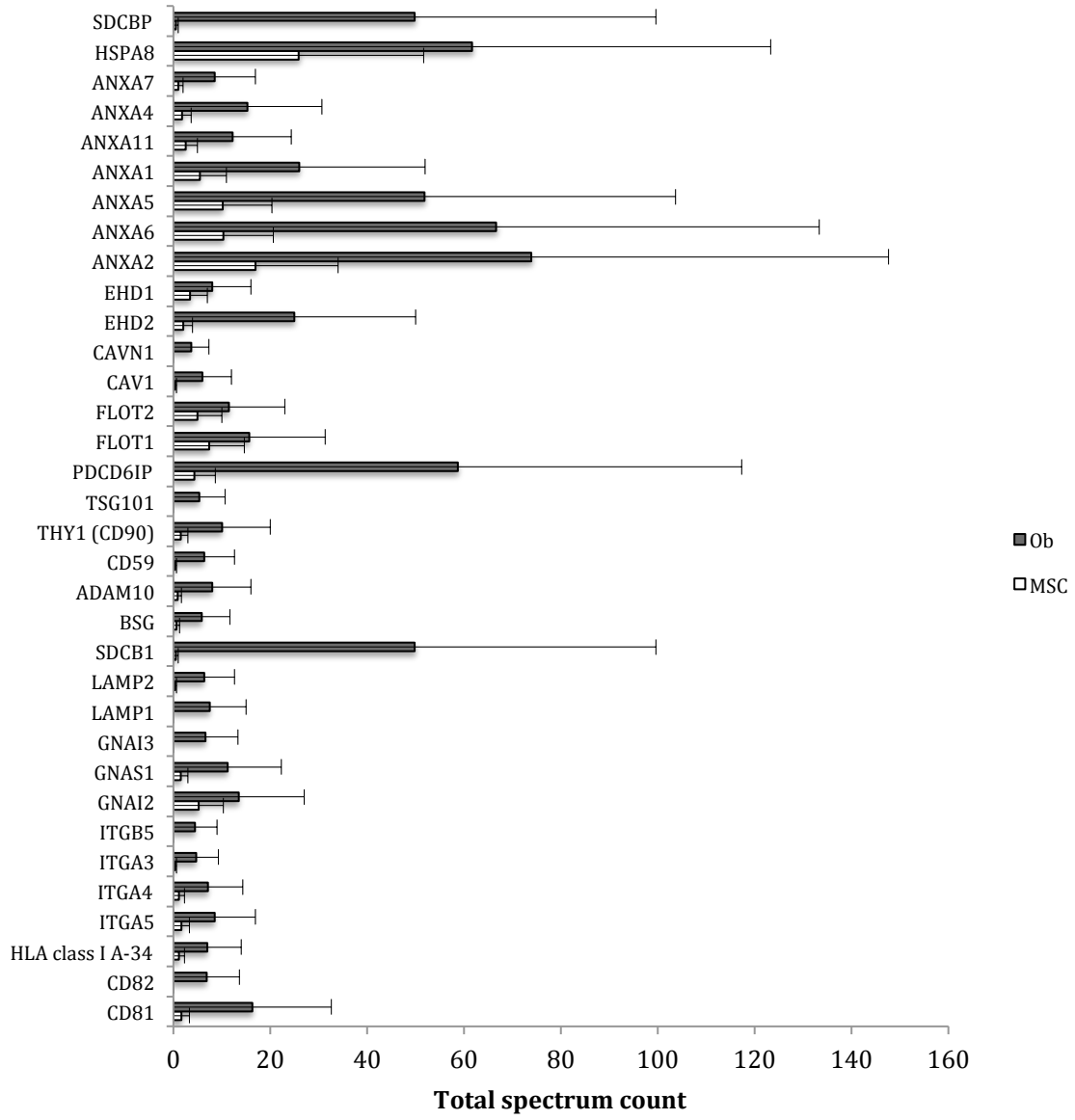

Figure 3.8: EV protein markers upregulated in Ob-EVs relative to the MSC-EVs

This bar chart demonstrates the average total spectral count (± standard deviation) of 34 EV protein markers that were exclusively or significantly expressed in Ob-EVs relative to MSC-EVs. All of these identified proteins had a *p*-value≤ 0.05. Abbreviations- EV: Extracellular vesicles; MSC: mesenchymal stem cells; Ob: osteoblasts.

3.8 Obs' EVs protein marker analysis

We further examined the protein content of the EV preparations to identify which category 1 and 2 proteins are significantly upregulated in the four OI-Ob-EVs relative to the two HC-Ob-EVs groups. Using Fischer's exact test, we identified three different proteins: Integrin α 5 (ITGAV), Syndecan Binding Protein (SDCBP/ Syntenin-1) and SDCB1 (Figure 3.9).

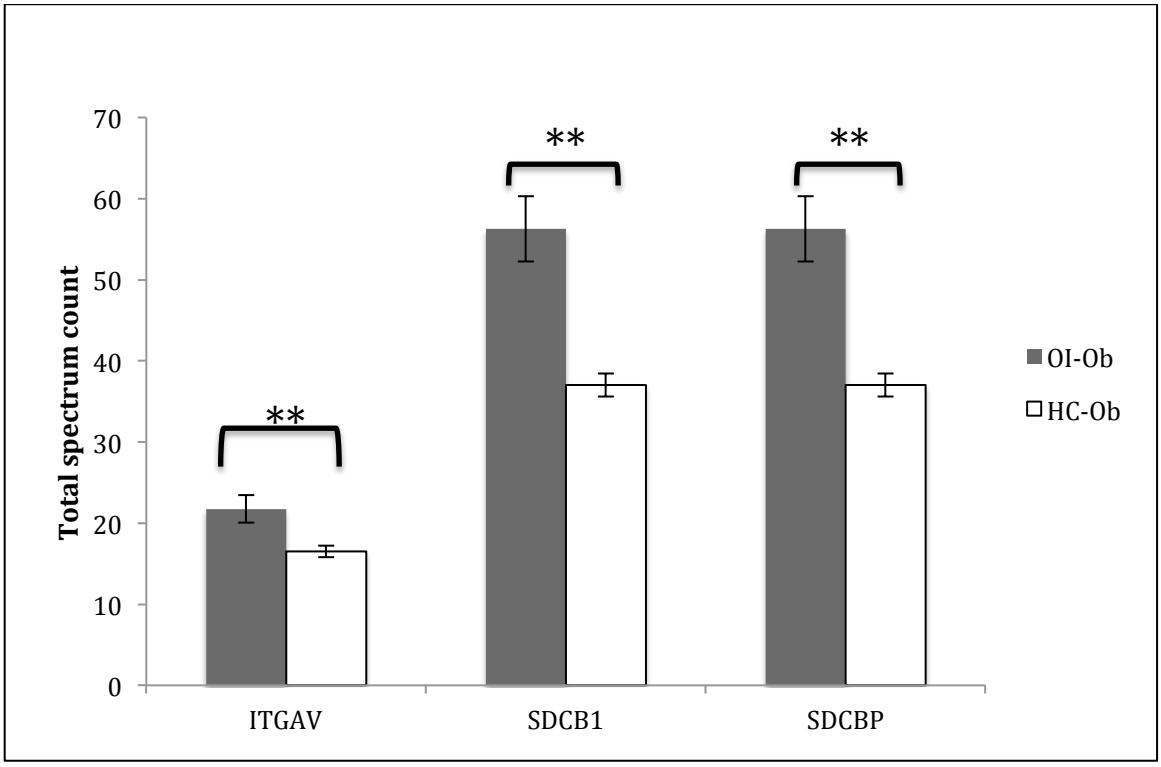

Figure 3.9: EV protein markers upregulated by the OI-Obs relative to the HC-Obs

ITGAV, SDCB1 and Syntenin-1 are EV protein markers that were significantly upregulated by the OI-Ob relative to the HC-Ob. This figure illustrates the average total spectral count of these three proteins in the EV groups ± standard deviation, where (**) represents a *p*-value≤ 0.01. Abbreviations- OI: Osteogenesis Imperfecta; HC: Human control; Ob: Osteoblasts; EV: Extracellular vesicles; ITGAV: Integrin α5; SDCBP: Syndecan Binding Protein (Syntenin-1) and SDCB1.

3.9 MSCs' EV protein marker analysis

We also analyzed and compared category 1 and 2 proteins markers in the OI-MSC and HC-MSCs groups using Fischer's exact test. We found no proteins that were exclusively or significantly (p-value ≤ 0.05) upregulated by either of these groups relative to other.

3.10 Comprehensive proteomic analysis of the osteoblasts' EVs

Because of our interest in OI-Ob behavior and the possible role they may have in the pathogenesis of the disorder, we wanted to compare the proteins expressed in EVs secreted by these cells relevant to the HC-Ob cells (i.e. analyze the protein content of the osteoblasts' EVs).

For this protein analysis, we conducted a Fischer's exact statistical test between the four HC-Ob-EV and two OI-Ob-EV groups of our study. As a result, from the total 384 proteins, we identified 95 proteins that were significantly (p-value ≤ 0.05) and differentially expressed by either of these groups relative to the other. 67 proteins were exclusively or significantly upregulated in OI-Ob-EVs, while 28 in the HC-Ob-EVs. Then we investigated the cellular component, molecular functions and biological processes of these two sets of proteins to aid us in understanding the significance of these differences in protein secretions by the two types of cells. To do so, functional enrichment analysis for Gene Ontology (GO) terms and comparison of the two data sets was done using FunRich (with human FunRich database as background; updated January 2019) (133). In order to show the highest diversity of enriched GO terms, we removed duplicate or highly similar terms and selected those with the highest statistical power.

Cellular component analysis, which assesses the cellular origin of a protein, showed that for both sets of protein, the most common origin was extracellular exosomes at 75% in the HC and at 76% in the OI group. They also exhibited other similarities such as both groups had

proteins originating from the extracellular space and extracellular matrix. However, there were differences in the origins of some proteins, for instance, plasma membrane was the second most common origin of proteins in the OI group but not a significant origin of the HC proteins (Figure 3.10).

Recall that these HC- and OI-Ob proteins were identified in EVs isolated from the culture medium of osteoblasts mineralizing the ECM. Having said that, molecular function analysis showed marked discrepancies and differences in the functions exhibited by these two sets of proteins. Proteins from the HC-Ob-EVs were exhibiting calcium-related activities, such as promoting calcium ion binding, calcium-dependent protein binding and calcium-dependent phospholipid binding (Figure 3.11). These are functions that are normally done by osteoblasts during the extracellular matrix mineralization. On the other hand, OI-Ob, instead of exclusively performing similar mineralization and calcium-related functions, they were predominantly involved in extracellular matrix formation functions such as ECM structuring and conferring elasticity, collagen binding, integrin binding and proteoglycan binding (Figure 3.11).

Analysis of the secreted HC-Ob proteins revealed their involvement in a single biological process, which was negative regulation of sequestering of calcium ion. This biological process matched the molecular functions performed by these proteins with an overall promotion of calcium release and preventing its confinement; thus, allowing the maintenance of calcium-related activities and functions. On the other hand, analysis of the biological processes of OI-Ob-EVs proteins further re-portrayed a similar impression of predominant ECM organization to the one seen with molecular function analysis. Interestingly, some of these proteins were also involved in extracellular matrix disassembly (Figure 3.12).

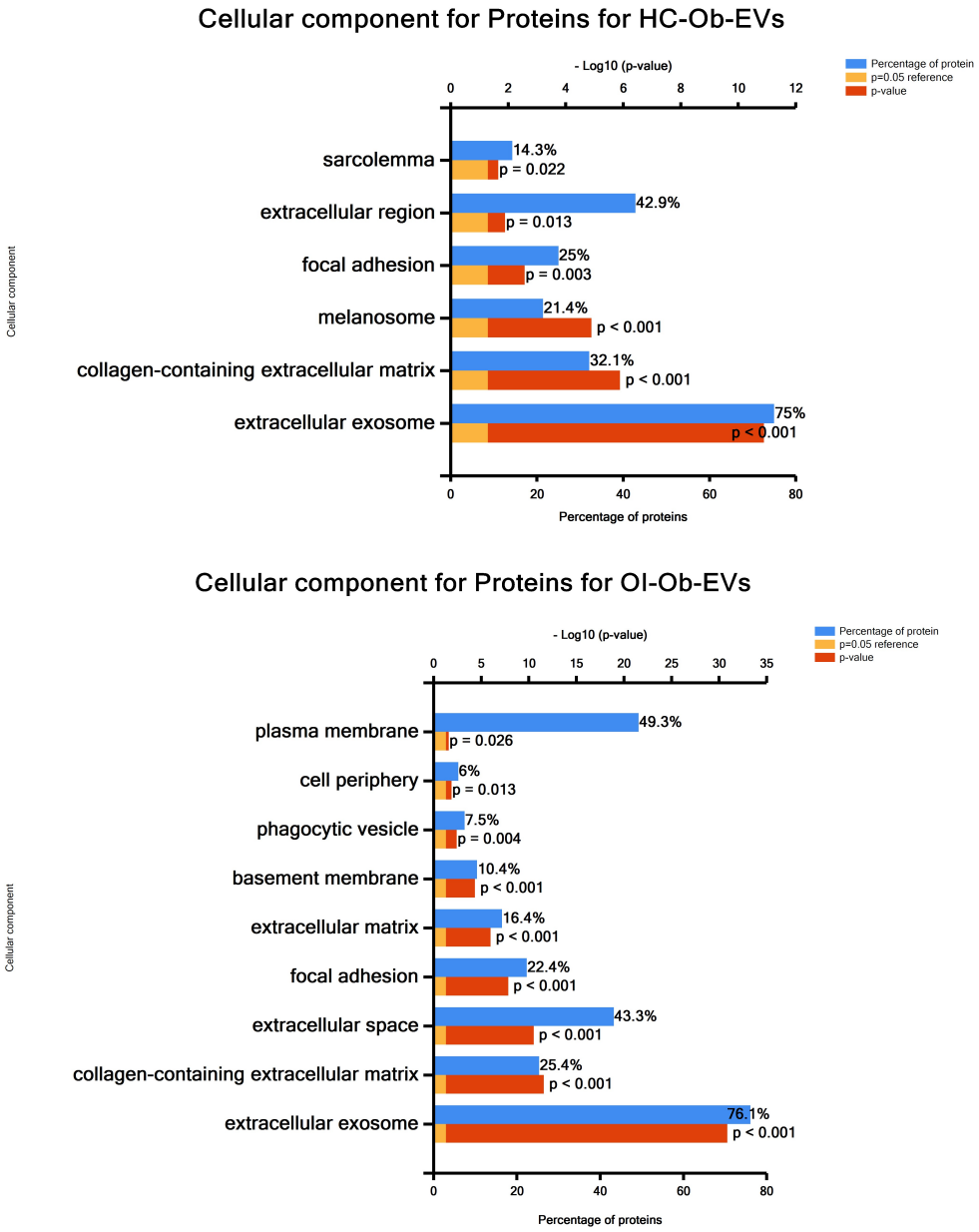

Figure 3.10: Cellular component GO enrichment analysis of the Obs-EVs

GO enrichment analysis for the cellular components of the HC- and OI-Ob-EVs proteins was performed and displayed on a bar chart using FunRich software. It revealed that for both sets of proteins, the most common origin was extracellular exosomes, but otherwise, had significant differences and variable origins. Abbreviations- HC: Human Control; OI: Osteogenesis Imperfecta; Ob: Osteoblast; EV: Extracellular vesicles; GO: Gene Ontology

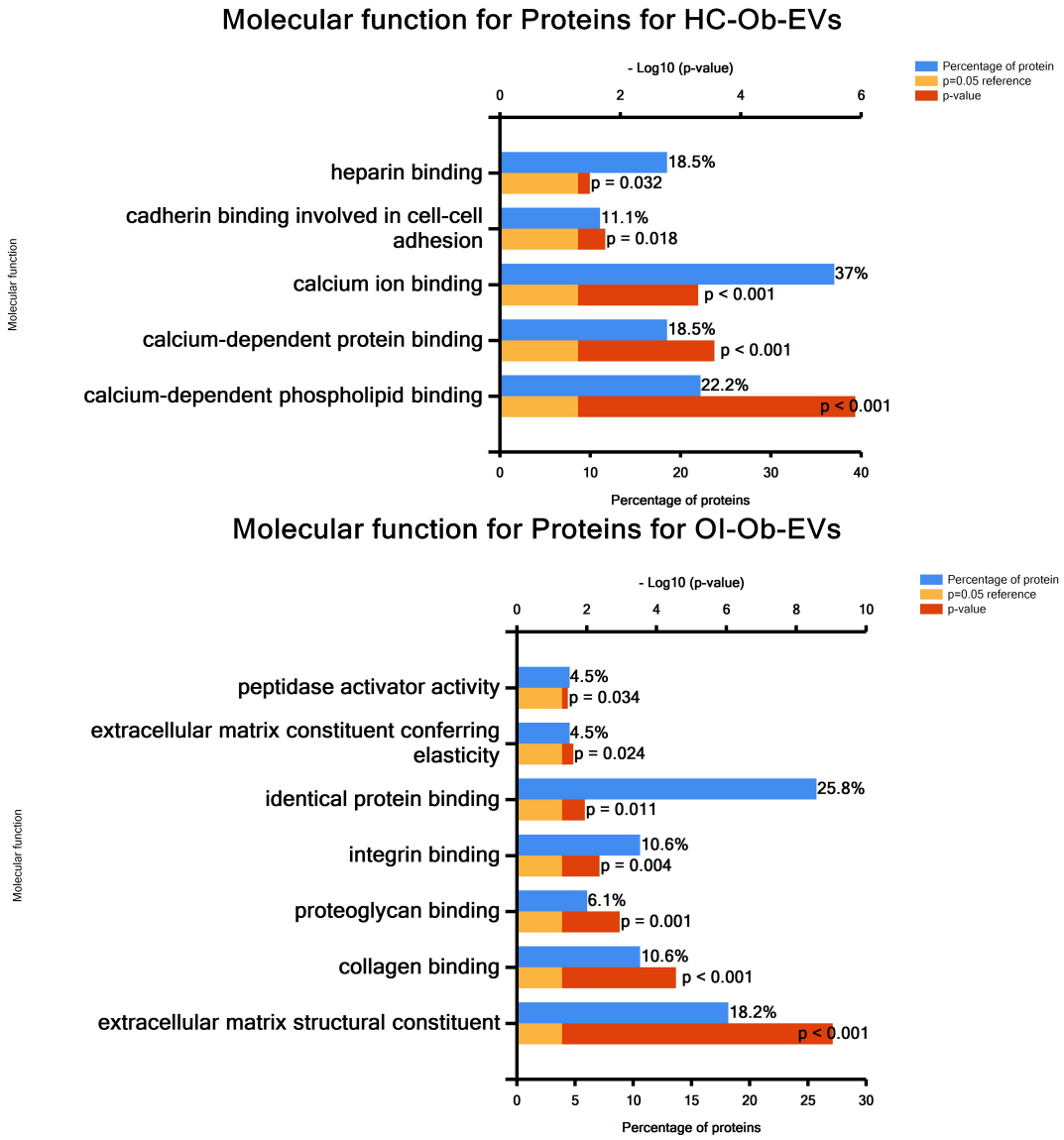

Figure 3.11: Molecular function GO enrichment analysis of the Obs'-EVs

GO enrichment analysis for the molecular functions of the HC- and OI-Ob-EVs proteins was performed and displayed on a bar chart using FunRich software. Analysis showed marked differences; HC-Ob-EV proteins were involved in calcium-related functions, whilst OI-Ob-EV proteins were predominantly involved in ECM structuring and protein-binding functions. Abbreviations- HC: Human Control; OI: Osteogenesis Imperfecta; Ob: Osteoblast; EV: Extracellular vesicles; ECM: Extracellular Matrix; GO: Gene Ontology

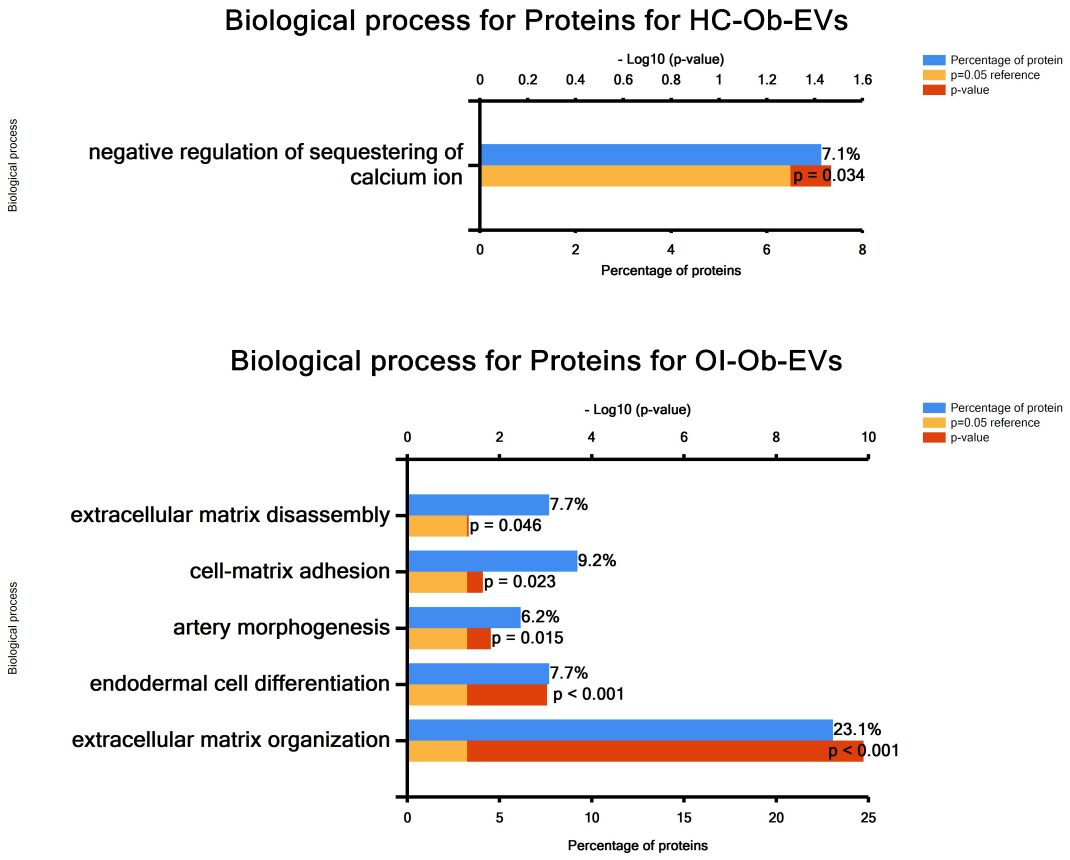

Figure 3.12: Biological process GO enrichment analysis of the Obs'-EVs

GO enrichment analysis for the biological processes of the HC- and OI-Ob-EVs proteins was performed and displayed on a bar chart using FunRich software. This analysis revealed the two sets of proteins were involved in different biological processes. HC-Ob-EV proteins were only involved in the negative regulation of sequestering of calcium ions. While, the OI-Ob-EV proteins were predominantly involved in ECM organization, as well as its disassembly. Abbreviations- HC: Human Control; OI: Osteogenesis Imperfecta; Ob: Osteoblast; EV: Extracellular vesicles; ECM: Extracellular Matrix; GO: Gene Ontology.

Of the 95 proteins that we have identified to be exclusively or significantly upregulated by either HC-Ob or the OI-Ob relative to the other, several proteins were of interest to us. Using Fischer's exact statistical test between the two HC-Ob-EV and the four OI-Ob-EV groups, we found five types of annexins to be differentially and significantly expressed. Annexins A6, A5, A2, A1 and A4 were significantly upregulated in the HC-Ob-EVs relative to the OI-Ob-EVs (Figure 3.13).

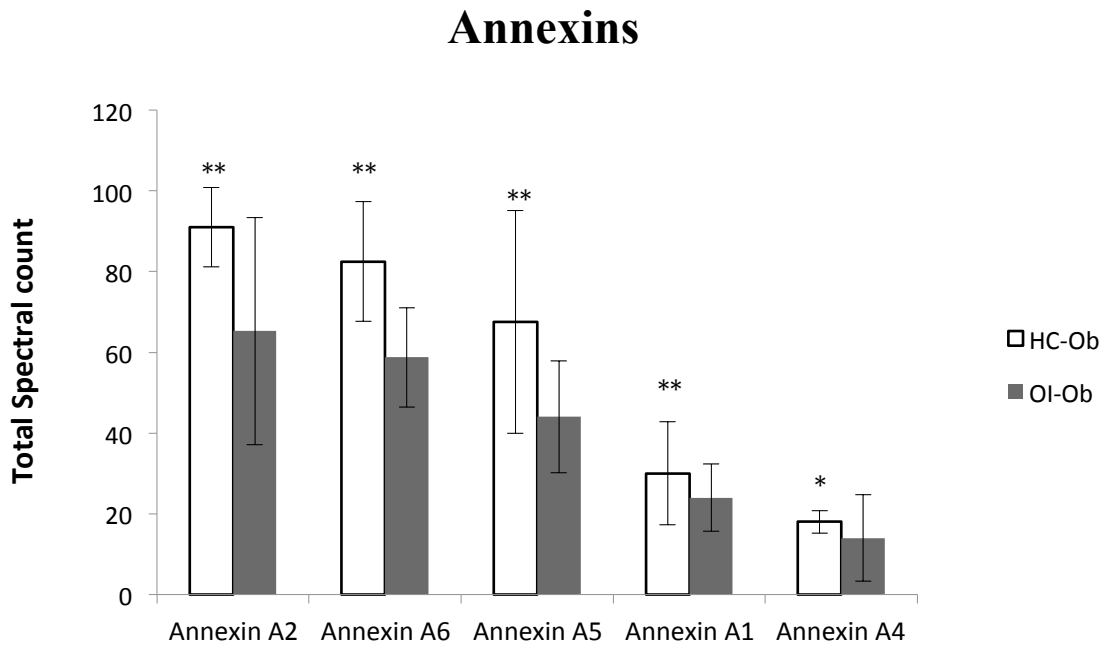

Figure 3.13: Annexins differentially upregulated in the HC-Ob-EVs

Annexins A1, A2, A4, A5 and A6 were significantly upregulated in the HC-Ob-EVs relative to the OI-Ob-EVs, as calculated by Fischer's exact statistical test. This figure illustrates the average total spectral count of each of these annexins found in the HC- and OI-Ob-EV groups ± standard deviation, where (**) represents a p-value$\leq$ 0.01 and (*) a p-value $\leq$0.05. Abbreviations- HC: Human control; OI: Osteogenesis Imperfecta; Ob: osteoblast; EV: extracellular vesicles

Using Fischer's exact statistical test between the two HC-Ob and the four OI-Ob- EV groups, eight extracellular matrix (ECM) proteins constituents were differentially and significantly upregulated (Figure 3.14). Seven ECM proteins were differentially upregulated in the OI-Ob-EVs relative to the HC-Ob-EVs, and they were fibronectin, nidogens-1, laminins (α4 and γ1), Fibulin-1, emilin-1 and heparan sulfate proteoglycan collagen. Conversely, we found α1 chain of type-1 collagen (COL1A1) to be differentially upregulated in the HC-Ob-EV relative to OI-Ob-EVs. This last finding goes along with what is expected and validates the nature of the OI genotype of our samples, in which there is a decreased production of the α1 chain of the type-1 collagen.

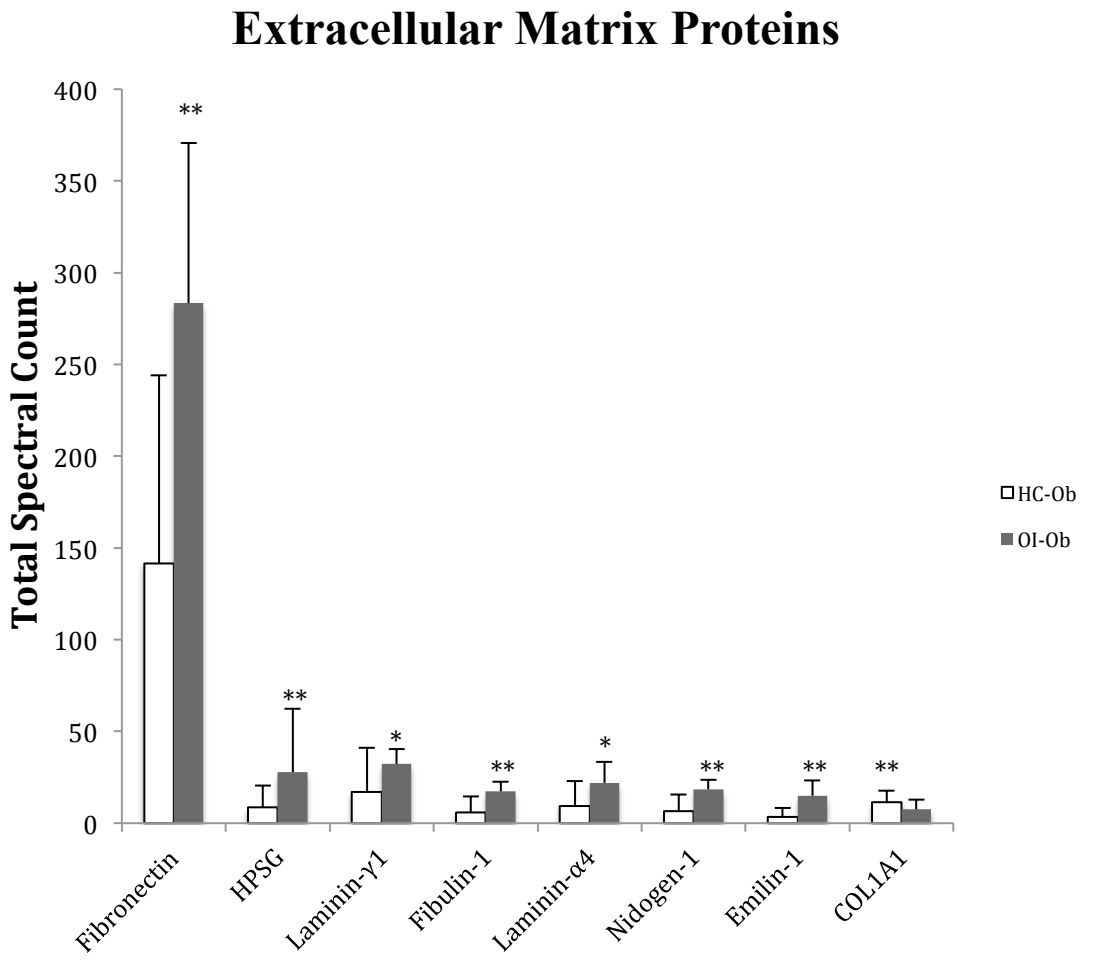

Figure 3.14: Differential expression of ECM proteins in the HC- and OI-Obs-EVs

Eight ECM proteins were differentially and significantly upregulated by one of the HC- and the OI-Ob-EV groups, relative to the other. COL1A1 was differentially upregulated in the HC-Ob-EVs, whilst the other seven non-collagenous proteins were differentially upregulated in the OI-Ob-EVs. This figure illustrates the average total spectral count of these eight proteins in these two groups ± standard deviation, where (**) represents a p-value$\leq$ 0.01 and (*) a p-value $\leq$0.05. Abbreviations- ECM: Extracellular matrix; COL1A1: Type I collagen α1 chain; HSPG: Heparan Sulfate Proteoglycan; HC: Human Control; OI: Osteogenesis Imperfecta; Ob: Osteoblast; EV: extracellular vesicles

Using Fischer's exact statistical test between the two HC-Ob-EV and the four OI-Ob-EV groups, four proteins were significantly upregulated in the OI-Ob-EVs relative to the other group. They are: High temperature requirement A1 (HtrA1), Matrix Metalloproteinase-14 (MMP-14), metalloreductase STEAP4 and cathepsin B (Figure 3.15).

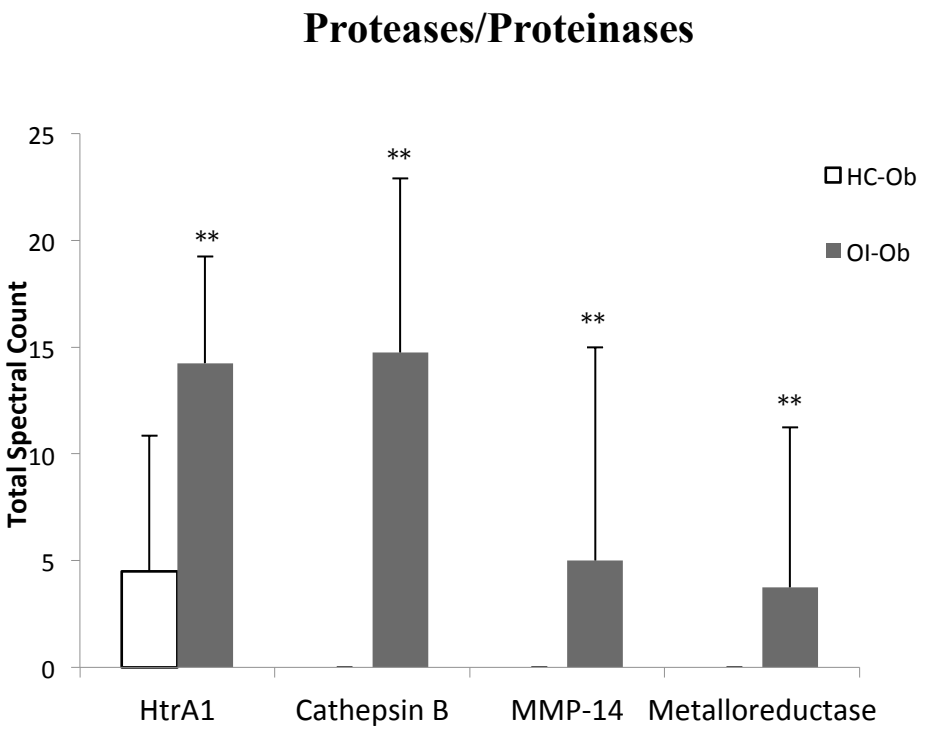

Figure 3.15: OI-Obs up regulate proteases/proteinases

The OI-Ob-EVs exclusively or significantly upregulated four different proteases/proteinases relative to the HC-Ob-EVs. This figure demonstrates the average total spectral count of these four proteins in the two groups ± standard deviation, where (**) represents a p-value$\leq$ 0.01. Abbreviations- HC: Human control; OI: Osteogenesis Imperfecta; HtrA1: High temperature requirement A1; MMP-14: Matrix Metalloproteinase-14.

Chapter 4: Discussion

Bone is an important component of the skeletal system that serves a wide range of mechanical, metabolic and hematopoietic functions (1). The extracellular matrix of bone provides elasticity and flexibility to it, whilst, its mineralization is what gives bone it's mechanical rigidity and load-bearing strength. Extracellular matrix mineralization is a process that is largely dependent on an orderly sequence of events that result in a mature, well-organized extracellular matrix (134, 135). Osteoblasts function to synthesize a proteinaceous extracellular matrix that is rich in collagens, predominantly type I, as well as other non-collagenous proteins. They then mineralize it by depositing calcium phosphate minerals through the expression of several calcium- and phosphate-binding proteins that help regulate this ordered process.

Given that osteoblasts function by paracrine signaling (72), we opted to study the behavior of osteoblasts of OI patients by comprehensively analyzing the protein content of their secreted extracellular vesicles. The aim of this study was to detect any deviation of OI-osteoblast behavior from what is expected or deemed normal as compared to HC-osteoblasts; thus, enhance our understanding of the pathophysiology of this disorder, particularly at a cellular level.

Nanoparticle tracking analysis of our EV groups showed an average size distribution and a mode particle size (i.e. most frequently detected size) of <300nm, which is consistent with previously published size of bone cells-derived vesicles (136). We also noted that the Ob significantly secreted more EVs relative to the MSCs, which reflects the functional and secretory nature of these cells. It is vital to keep mind that NTA has its limitations, such as its inability to distinguish between large EVs and aggregated EVs that result from the high-speed

ultracentrifugation during EV isolation (137). For this reason, we have analyzed the EV size distribution and concentration detected by NTA with conservation.

In our EV protein markers analysis, we followed the International Society of Extracellular Vesicles' (ISEV) recommended list of proteins (94). They divided this list into category 1 and 2 proteins, which are transmembrane or GPI-anchored proteins and cytosolic or periplasmic proteins respectively. We met the ISEV's minimum requirement of identifying at least one protein from each of these categories in all of our samples, thereby demonstrating the presence of EVs in all of them. ISEV also published a list of proteins (category 3) that are constituents of common non-EV structures often co-isolated with EVs. Of these, we noted albumin to be present in all of our EV preparations. We attributed this finding to the use of 10% FBS in our cell culture medium. From our expertise, 10% FBS supplementation of the growth and osteogenic medias is necessary to achieve optimal MSCs proliferation, Obs differentiation and ECM mineralization. For this reason, we believe it is reasonable to use 10 % FBS in our experiments at the expense of having significant amounts of albumin contamination. We also believe this is a minor concern, as it does not interfere with the mass spectrometry analysis.

Using the category 1 and 2 EV protein markers, we conducted several analyses and comparisons between our EV groups. We identified eight different proteins that were found in all EV groups: HC-MSCs, HC-Ob, OI-MSC and OI-Ob. Interestingly, two of these eight proteins were heat shock proteins, HSP70 and HSP90, that are thought to be exosomal markers (138-140). However, when it comes to attributing a protein marker to a specific subtype of EV, there is still plenty of debate. As the EV field advanced, the techniques by which EVs are isolated

have changed and newer ones were introduced. It is believed that different EV isolation approaches and different cellular sources yield different EV protein markers/compositions (94, 101-104). This has raised concerns over the accuracy of what was previously considered a specific and universal marker of one or the other types of EVs. It is for this reason that the ISEV has reached a consensus that it may still not be possible to propose such protein markers (94). As such, we will be speculating and examining what has been previously proposed as protein markers for a specific EV subtype conservatively and with caution.

We also analyzed the differential expression of EV protein markers between the MSCs and Obs. We were able to identify 34 different markers that were differentially and significantly upregulated by the osteoblasts' EVs (p-value ≤ 0.05). Nine of the 34 proteins were HSPA8/70, CD81, CD82, Syntenin-1, flotillin-1 (Flot-1), TSG101, ADAM10, CAV1 (Caveolin-1) and PDCD6IP (Alix). These are thought to be exosomal markers (85, 141-146), which may imply that our Obs secreted more exosomes than the MSCs. This of course may be in part due to the fact that the Obs secreted more EVs in total than MSCs, as we have seen with their significantly greater EV concentration relative to the MSCs. On the other hand, there were no differentially and significantly upregulated EV protein markers between the HC-MSC and OI-MSC, which may imply that the type of EVs secreted by these cells is similar.

Interestingly, we identified three EV protein markers that were significantly upregulated by the OI-Obs relative to the HC-Ob: Integrin α5 (ITGAV), Syndecan Binding Protein (SDCBP/ Syntenin-1) and SDCB1. This raises the possibility of using one of these proteins as a specific marker of OI-Ob-EVs, however further analysis and comprehensive studies need to be done to assess their sensitivity and specificity for OI-Ob-EVs.

After analyzing the protein markers of our EV samples and their possible heterogeneity, we then conducted a comprehensive analysis of the full proteomic content of the EVs. We identified 384 proteins, 373 (97%) of them were found in Vesiclepedia. This high percentage indicates a very good enrichment of our samples with EV proteins. This validates the nature of the studied samples to be rich in EVs and the success of our EV isolation, enrichment and concentration protocol. These proteins mostly originated from extracellular exosomes, but also from several other cellular origins as revealed by their cellular component GO enrichment analysis. The 11 proteins not found in Vesiclepedia are likely to be unique to our samples and/or never encountered before in EVs.

We then compared the proteomic content of the OI- and HC-Ob-EVs, and identified two sets of proteins that are differentially and significantly upregulated in one of these two EV groups relative to the other. To highlight the differences between these two sets of proteins and the significance of that, we did cellular component, molecular function and biological processes Gene Ontology enrichment analyses.

The cellular component enrichment analysis of these two sets of proteins revealed that the far majority of the proteins were of extracellular exosomes in origin. However, there were significant differences in the cellular origins of the remaining proteins. This may imply that these two types of cells, OI-Ob and HC-Ob, have proteins originating from different components/parts of the cell; thus, different biogenesis of the packaging EVs and so different subtypes. For instance, one study analyzed and compared the RNA content of normal breast cells and compared them to that of malignantly transformed breast tumor cells (78). They showed that there were major differences in the EV subpopulations secreted by these two types of cells. In

our study, OI-Ob may be considered a transformed 'genetically mutated' cell and so predicted to have different EV subpopulation from the healthy HC-Obs.

From our expertise, numerous published studies and from the HC –cells that we simultaneously cultured, 14 to 21 days of osteogenic treatment induction is expected to induce MSCs to differentiate into mineralizing osteoblasts. These mineralizing osteoblasts are expected to be predominantly or exclusively mineralizing their surrounding ECM and conducting other similar calcium-related functions. These anticipated functions were clearly exhibited by the normally behaving human control-osteoblasts, as was displayed by molecular function and biological process analysis of their EV-proteins.

On the other hand, the OI-osteoblasts were not behaving similarly and this was portrayed by the significant difference in the protein content of their secreted EVs. These proteins were assessed for molecular functions and biological processes, which revealed predominant extracellular matrix organization with collagen-, proteoglycan-, integrin- and other protein-extracellular matrix binding functions. These are important functions done by osteoblast to form, organize and mature the bone extracellular matrix; however, they are done at a very early stage shortly following osteogenic treatment initiation. In other words, this has led us to hypothesis that the persistence of these functions following 14-21 days of osteogenic treatment may indicate a lag or hindrance in the progression of the normal process of osteoblast functioning from predominant ECM maturation/organization (earlier stage) to predominant ECM mineralization (later stage). This may also indicate the presence of a structurally altered ECM of the OI osteoblasts, which is what may be contributing to this interruption that we hypothesize is occurring.

This alteration in the organic and inorganic bone matrix composition in OI has been reported in numerous studies (147-150). Our hypothesis was also previously proposed by a study assessing the protein composition of extracellular matrix of osteogenesis imperfecta patients (147). As expected with autosomal dominant OI, which affects the production of type I collagen, the ECM protein composition of these OI cells was found to be low in type I collagen. Conversely, they found that levels of non-collagenous proteins, especially fibronectin, were significantly elevated when compared to age-matched controls. They hypothesized that this alteration in the levels of matrix proteins, which are vital in modulating ECM organization and mineralization, contribute, at least partially, to the bone fragility phenotype seen in OI. They also stated that the OI matrix composition is immature and in that respect, comparable to a fetal bone matrix that is also low in type 1 collagen and high in non-collagenous proteins.

Similarly, when looking at our differentially expressed protein analysis of our OI- and HC-Ob-EVs, several non-collagenous proteins were significantly upregulated by the OI group. Fibronectin, nidogen-1, laminins (α4 and γ1), Fibulin-1, emilin-1 and heparan sulfate proteoglycan are extracellular matrix proteins (10, 147, 151, 152). They are integral in achieving and maintaining the structural and functional integrity of the ECM. We speculate that this constitutive expression of ECM proteins may indicate a persisting (unsuccessful) attempt of organizing the extracellular matrix, which is relatively deficient in normal type I collagen. The presence of a dysfunctional/abnormal ECM in OI; therefore, compensates its mineralization and compromises the property and integrity of the bone in OI (50, 147).

Interestingly also, in our comprehensive analysis of the biological processes of the OI-Ob-EV differentially up regulated proteins, we noted that some of them were involved in extracellular matrix disassembly. This finding gave us a complementary perspective on how OI-

Ob could possibly be contributing to OI's bone fragility phenotype. We identified several of these proteins to be possible culprits in this speculated pathogenesis, and they were: HtrA1, metalloreductase STEAP4, matrix metalloproteinases-14 and cathepsin B.

High temperature requirement protease A1 (HtrA1) is a secretory serine protease (i.e. degrades proteins) that has several substrates in the extracellular matrix (153-156). In numerous *in-vivo* and *in-vitro* studies, HtrA1 has been shown to inhibit mineralization and bone formation by cleaving and antagonizing ECM proteins important for promoting osteogenic differentiation and mineral deposition, such as TGF-β and BMP (153-155, 157, 158). In addition, HtrA1 can also degrade osteoprotegerin (OPG) found in the ECM (156). OPG is an inhibitor of RANKL, which is a key regulator of development, activation and differentiation of osteoclasts (osteoclastogenesis) (49, 123, 124). Thus, by removing OPG's inhibition of RANKL, HtrA1 promotes the bone resorption phase of bone remodeling. Similarly, metalloreductase STEAP4 is a ferric-chelate reductase that is critical for cellular iron uptake and utilization in osteoclasts, hence promoting its development and function (159).

Bone remodeling is the process by which bone is constantly renewed to ensure maximization and maintenance of its health and functionality (1). An essential phase in this process is bone resorption, which is orchestrated and conducted by osteoclasts. Degradation of the extracellular matrix is considered a principal feature of this phase, and this process is thought to be regulated by several proteinases, such as matrix metalloproteinases (MMP) and cysteine proteases (cathepsins) (160, 161). MMP14, also called membrane-type 1 MMP (MT-1 MMP), and cathepsin B are proteinases that belong to these two families of lysosomal enzymes normally secreted by osteoclasts. MMP14 can degrade several non-collagenous ECM proteins, including fibronectin, vitronectin and laminin (162), as well as type 1 collagen (163). It also aids

(pre)osteoclasts in migrating and anchoring onto bone surfaces where bone resorption is to be initiated (164). Similarly, cathepsin B is an osteoclastic lysosomal protease that degrades type 1 collagen among other ECM proteins (165-167). In addition, its inhibition has been shown to limit tumor progression and bone metastasis by preventing cells from invading through the ECM and stimulating angiogenesis (165, 168).

As alluded to, all of these proteins serve vital functions in maintaining a healthy bone. However, our OI-Obs, despite being adequately stimulated and induced to enter osteogenesis and explicitly do ECM formation- and mineralization-related functions, are instead secreting proteins that are not necessarily/directly useful for these functions. This suggests that in OI, the causative mutation may be resulting in an impaired Ob behavior diverging them away from committing to osteogenesis and instead promoting alternative pathways that may contribute to the OI phenotype.

To further understand what are the direct and indirect effects of the OI mutations on bone development, bone histomorphometry studies in OI children were conducted (58). Histomorphometry is the study by which the behavior of bone cells is analyzed within the *in-vivo* structural context. In normal bone remodeling, there is a balanced alternation between the osteoblastic-bone formation and osteoclastic-bone resorption cycles (1). However, in the bone histomorphometry studies, they found that the OI patients have a lower wall thickness when compared to healthy controls. This was attributed to their finding of OI-osteoblasts producing lesser amounts of matrix per unit time during a remodeling cycle relative to the HC-osteoblasts. In addition, they found that the number of bone turnover cycles on a given bone surface per unit time (i.e. bone turnover rate) and the frequency of osteoclast recruitment were both increased. Therefore, these findings suggest that in OI a single genetic defect could result in decreased bone

mass, the characteristic feature of OI, by several mechanisms, the main one of which is an imbalance in bone remodeling.

Within the bone microenvironment niche, osteoblasts and osteoclasts synthesize and secrete paracrine-signaling biomolecules to maintain the architecture of the skeleton (71, 169-173). In other words, EV-packaged proteins secreted by bone cells can regulate osteoblastogenesis and osteoclastogenesis. For this reason, we postulate that some of the OI-Ob-EV proteins that we have highlighted in our study may have a role in contributing to the above reported *in-vivo* bone remodeling imbalances that occur in OI. It would therefore be quite interesting to investigate the paracrine behavior of OI-derived osteoclasts, as well as the effect of the OI-Ob-EV proteins on osteoclastogenesis.

Despite bisphosphonates being the gold standard of medical management of OI, there is limited evidence of their benefit. Several meta-analyses have shown that bisphosphonates neither significantly reduce the overall rate of long bone fractures (59, 60), nor significantly improve patient functioning (61). For this reason, an alternative better medical management is a clinical need that is yet to be met. Our finding of OI-Ob secreting several proteins that may be culprits in inhibiting ECM mineralization and/or promoting its degradation/resorption raises the opportunity of developing future drug therapies aimed at one of them to limit/reverse their probable undesirable effects in OI patients.

Besides secreting the aforementioned proteins and their hypothetical undesirable roles in OI, we have noted that OI-Ob deviate from normal behavior in other ways too. One of our working hypotheses prior to starting this study was that OI-Ob might have some form of ECM mineralization-loss of function that occurs as a result of the OI mutation. This hypothesis was supported with our analysis of the differentially expressed proteins between the OI- and the HC-

Ob-EV groups. We noted that the OI-Ob-EVs had significantly lower expression of five different types of annexins relative to the HC-Ob-EVs, including annexins (Anx) A1, A2, A4, A5 and A6.

Numerous studies have shown that these different types of annexins all have integral roles in various processes necessary for ECM mineralization and bone development. In general, the fundamental biochemical characteristic of all annexins is their calcium-affinity and calcium-dependent binding to the phospholipids in membranes. This makes them influential in several processes such as endocytosis, exocytosis, membrane fusion, calcium channel formation, and cytoskeleton-cytoplasmic face of cell membranes interface organization (174-177). In bone, AnxA1 is important for the osteogenic differentiation of MSCs into Obs, and an AnxA1 knockout mouse model has exhibited delayed intramembranous ossification (178). AnxA2 and AnxA5 are also important for proliferation and differentiation of osteoblasts and matrix maturation and mineralization, as concluded from AnxA2- and AnxA5-knockdown pre-osteoblast studies (179). AnxA2 are also thought to facilitate the matrix mineralization process by enhancing ALP activity (180). Annexins, particularly A2, A5 and A6, are also thought to be integral in forming calcium channels to supply high concentrations of it to the ECM for mineralization (calcium phosphate deposition)(26). Therefore, the pattern of significantly lower expression/downregulation of these annexins in OI-Ob-EVs may thus be a cause of the decreased bone volume and attenuated osteoblast functioning that was reported in the OI bone histomorphometry studies (58).

Given that our study cells were of OI patients with a mutation in the *COL1A1* gene, our findings and interpretations are thus limited to this genotype. As such, it would be intriguing to investigate the proteomic paracrine behavior of cells from the other types of OI with different mutations/affected genes, and see how they compare to the findings of this study.

Chapter 5: Conclusion

In this research project, our aim was to broaden our knowledge of OI pathogenesis by investigating osteoblasts of *COL1A1* OI patients, and their paracrine behavior. We analyzed the protein content of OI-Ob-EVs relative to the HC-Ob-EVs, focusing on their differentially and significantly expressed proteins. As a result, we found a persistent elevation of several non-collagenous ECM proteins in the OI-Ob-EVs such as fibronectin, laminins and HSPG, which may imply the presence of a dysfunctional and hindrance in ECM organization. There was also a significant relative under-expression of several annexin proteins which are vital for ECM maturation and mineralization. Interestingly also, there was an overexpression of several proteases and proteinases that are involved in inhibiting ECM mineralization and instead promoting its degradation. In conclusion, these findings altogether, suggest that a single genetic mutation carried in an OI-osteoblast may be resulting in impairment in several bone development processes, and thus, contributing to OI's bone manifestations and characteristics.

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