Periosteum-Derived Mesenchymal Stem Cells Secretome - Cell-Free Strategy for Endogenous Bone Regeneration: Proteomic Analysis in Vitro

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ABSTRACT

Objectives: Millions of people worldwide are affected by diseases or injuries which lead to bone/tooth loss and defects. While such clinical situations are daily practice in most of the hospitals, the widely used treatment methods still have disadvantages. Therefore, this field of medicine is actively searching new tissue regeneration techniques, one of which could be stem cell secretome. Thus, the purpose of this research study was to perform the detail proteomic analysis of periosteum-derived mesenchymal stem cells secretome in order to evaluate if it is capable to induce osteo-regenerative process.

Material and Methods: Periosteum-derived mesenchymal stem cells (PMSCs) were extracted from adult male New Zealand White rabbits. Cells were characterised by evaluating their differentiation potential. After characterisation PMSCs secretomes were collected and their proteomic analysis was performed.

Results: PMSCs were extracted from adult male New Zealand White rabbits. In order to characterise the extracted PMSCs, they were differentiated in the directions which mainly describes MSC multipotency - osteogenic, myogenic and adipogenic. A total of 146 proteins were detected. The resulting protein composition indicates the ability to promote bone regeneration to fully mature bone.

Conclusions: Bioactive molecules detected in periosteum-derived mesenchymal stem cells secretome initiates the processes required for the formation of a fully functional bone.

Keywords: bone regeneration; mesenchymal stem cells; periosteum.

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INTRODUCTION

Periodontal diseases and face injuries have led to bone and tooth loss and defects, which have become a global concern, often affecting the health and quality of life of the entire population and placing a heavy financial encumbrance on community [1,2]. Such bone defects regeneration can be defined as a complex mechanism based on the interaction between osteogenic, angiogenic, chondrogenic, etc processes able to drive bone growth and tissue restoration [3-5]. During bone defect regeneration process, different cell lineages interact with each other in order to promote tissue healing. In novel bone development, osteogenic, angiogenic, and neurogenic processes are closely connected [6,7]. The blood vessels of bone tissue can transport minerals and growth factors and, at the same time, represent the physical structures around which bone deposition start [8,9].

Over the past decade, there has been a growing interest in the therapeutic application of autologous products/stimulants such as platelet rich fibrin (PRF), platelet rich plasma (PRP), plasma rich in growth factors (PRGF), mesenchymal stem cells (MSCs) etc for the regeneration/treatment of bone defects [10-12]. For multipotency and ability to differentiate into osteogenic cell lineage, stem cell-based therapy is assessed as one of the most perspective technique in bone regenerative medicine [13]. Even though cell-based therapies including injection or transplantation of MSCs are promising strategies, some concerns remain, such as technical limitations and low survival rates of transplanted cells [14]. Furthermore, some studies report increase in apoptosis after transplantation often triggers an immune response, resulting in worsening of the diseased condition or rejection of the transplanted cells [15,16]. Recent studies revealed that bone marrow, stromal, hematopoietic MSCs can contribute to tissue regeneration not only through their multipotency but also by stimulating the recipient cells via paracrine mechanisms [17,18]. The paracrine effects are mediated by secretomes including cytokines and chemokines. As the secretomes from MSCs contain various factors exerting several biological effects, they are also expected to be applied clinically and provide novel strategies for regenerative medicine [19]. However, according to the studies different types of MSCs produces and secrets different bioactive molecules. For example, secretomes obtained of human brown and white adipogenic MSCs has different protein composition and according to that greater abundance of immunoreactive proteins were detected at in the secretome from brown adipogenic MSCs secretome compared to white adipogenic MSCs secretome [20]. Therefore, it was demonstrated that the origin of MSCs determined the proteomic profile of MSCs secretomes and predetermined it’s biological functions [21].

The origin of the MSCs not only depends the specific molecules which secret the cell, but also determines their differentiation potential [22,23]. For example, MSCs isolated from adipose tissue will more easily differentiate in the adipose direction compared to bone marrow derived MSCs and vice versa [24]. Meanwhile, osteogenesis would be more easily induced in MSCs derived from bone-adjacent tissues, such as periostea. For this reason and secretome produced by periosteal-derived MSCs (PMSCs) may be well suited for bone regeneration. However, protein composition and biological functions of PMSCs secretome has not been fully investigated yet.

Thus, in this research study, we aimed to characterize periosteum-derived mesenchymal stem cells secretome, in terms of their proteomic composition.

MATERIAL AND METHODS

Cell source

PMSCs were extracted from three randomly chosen adult male New Zealand White rabbits, weighing approximately 3 kg. Rabbits were used in this study with the approval of the State Food and Veterinary Service (identification code: G2-55). The study was conducted from February 1, 2017 to December 31, 2019. The rabbits were housed in a temperature-controlled room (21 to 23°C) and accommodated under a 12 h light-dark cycle. An individual cage was intended, and animal was fed by standard dried diet and water ad libitum. The premedication was induced by injection of acpromazine (0.5 mg/kg) (Temprace, Oudewater, Netherlands) in thigh muscles and a subcutaneous injection of buprenorphine (0.03 mg/kg) (INDIVIOR INC, North Chesterfield, USA). General anaesthesia was achieved by injection of ketamine hydrochloride (35 mg/kg) (Salfarm Danmark A/S, Kolding, Denmark) and xylazine hydrochloride (5 mg/kg) (Xylomed Pharmaceuticals Limited, Gloucestershire, UK) in thigh muscles. The Carbomer Eye Gel (Oftagel® 2.5 mg/g - SANTEN OY; Tampere, Finland) was used to keep the eyes wet. Surgical procedure was performed using a special warming surgical table and special cover to keep the animals warm and achieve better sterility. After shaven of the calvaria area, it was disinfected with alcohol and local anaesthetic with...
artificial and epinephrinum performed (Ubistesin™ forte [40 mg + 5 mcg/ml] - 3M Deutschland GmbH; Neuss, Germany). After preparation for operation, the surgical area was expanded using a sagittal incision through skin and periosteum around the entire thickness. A 5 x 5 mm of periosteum was cut out.

Isolation and cultivation of PMSCs

PMSCs were isolated from periosteum obtained from the calvaria site, as previously described [25]. Briefly, tissue was further processed under sterile laminar flow conditions. Using sterile scissors tissue was minced into smaller pieces, approximately 1 mm³ in size. Periosteum tissue pieces were transferred to the sterile 15 mL vial with 4 ml of 1 mg/ml collagenase A solution prepared in DMEM for 18 hours at 37 °C while gently stirring. Then, tissue pieces were separated from the cells by filtering through a sterile 70 µm nylon mesh sieve. The cell suspension was centrifuged at 400 g for 10 min at 4 °C, the supernatant was then discarded, and cells were suspended in growth media (GM) composed of Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics: penicillin 100 U/mL, streptomycin 100 mg/mL. PMSCs were sowed at 75 cm² Falcon flasks, at the density of 40,000 cells/cm². Later it was grown in GM. All growth supplements were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, Maryland, USA). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Subconfluent rPMSCs were trypsinized and used in subsequent experiments.

Evaluation of PMSC differentiation potential

In order to evaluate PMSCs differentiation potential, cells were grown in adipogenic, myogenic and osteogenic differentiation inducing media (Figure 1). For adipogenic differentiation induction, cells were grown in DMEM supplemented with 10% FBS, 1% penicillin streptomycin solution (Sigma-Aldrich Co.; Darmstadt, Germany), 1% glutamine (Sigma-Aldrich Co.; Darmstadt, Germany), 1% penicillin streptomycin solution, 10 mM β-glycerophosphate, 25 µg/ml ascorbic acid and 50 nM dexamethasone. Differentiation was carried out for 21 days, half of the media was replaced every two/three days. Adipogenic differentiation was confirmed by staining formed calcified extracellular matrix (ECM) with alizarin red S (ARS) dye. Osteogenic differentiation media was removed and cells were fixed with 4% formaldehyde for 10 min at room temperature. Then cells were washed 3 times with PBS and stained with 2% ARS (pH 4.1 - 4.2). Cells formed calcified ECM (stained in red) were visualized and captured with a CCD camera (EXi Blue™ - QImaging) attached to a microscope (Olympus IX51 - Olympus Co.). For osteogenic differentiation cells were grown in DMEM supplemented with 10% FBS, 1% penicillin streptomycin solution, 10 mM β-glycerophosphate, 25 µg/ml ascorbic acid and 50 nM dexamethasone. Osteogenic differentiation was confirmed by staining formed calcified extracellular matrix (ECM) with alizarin red S (ARS) dye. Osteogenic differentiation media was removed and cells were fixed with 4% formaldehyde for 10 min at room temperature. Then cells were washed 3 times with PBS and stained with 2% ARS (pH 4.1 - 4.2). Cells formed calcified ECM (stained in red) were visualized and captured with a CCD camera (EXi Blue™ - QImaging) attached to a microscope (Olympus IX51 - Olympus Co.).

Secretome preparation

PMSCs were seeded at the density of 40 000 cells/cm² in the 75 cm² flasks (Thermo Fisher Scientific, Inc., Waltham, USA). The next day, growth media was removed, cells were 3 times washed with PBS (Gibco™ - Thermo Fisher Scientific, Inc.) and serum-free DMEM was added. Cells were grown for 3 days in 37 °C with 5% CO₂ atmosphere and 95% humidity. After predetermined time the secretomes was collected to 50 ml tubes (Thermo Fisher Scientific, Inc.) and centrifuged for 15 min at 6000 RCF. Supernatant was filtered through 0.22 µm syringe driven PVDF filter (Thermo Fisher Scientific, Inc.) to new 50 ml tubes, and stored at 4 °C. All secretomes were used up in 30 days after collection.
Proteomic analysis

Sample preparation

Filter aided sample preparation (FASP) [26] method was used for protein digestion prior to mass spectrometry analyses. Protein lysates were processed by the FASP using Microcon® 30k centrifugal ultrafiltration units (Merck Millipore; Darmstadt, Germany) operated at 10,000 g. Briefly, the sample was diluted with 200 μL of 8 M urea (pH 8.5), placed in a filter unit, centrifuged and washed two times with 100 μL of 8 M urea. Then, 100 μL of 55 mM iodoacetamide was added to the filters, and samples were incubated for 20 min. Filters were washed twice with 100 μL of 8 M urea followed by two washes with 100 μL of 50 mM NH₄HCO₃ at pH 8.0. Protein digestion was then performed by adding trypsin in 50 μL of 50 mM NH₄HCO₃ at an enzyme to protein ratio of 1:100 and incubating overnight at 37 °C. Peptides were collected from the concentrators by centrifugation at 10000 g for 10 min and additionally eluted using 20% CH₃CN. The eluates were combined, acidified with 10% CF₃COOH and peptides were dried in a speed vacuum for 2 hours at 45 °C. The lyophilized peptides were redissolved in 0.1% formic acid.

LC-MS based protein identification

Liquid chromatographic (LC) analysis was performed in a Waters® Acquity® Ultra Performance LC system (Waters Co., Wilmslow, Manchester, UK). Peptide separation was performed on an Acquity® UPLC HSS T3 250 mm analytical column (Waters Co.). Data were acquired using Synapt G2

Figure 1. Evaluation of periosteum-derived mesenchymal stem cells differentiation potential: A, B = osteogenic differentiation lineage (original magnification x10); C, D = myogenic differentiation lineage (original magnification x40); adipogenic differentiation lineage (original magnification x40). Control column (A, C, E) - cells stained with the same dyes but were grown in growth media without differentiation inducing supplements, differentiated column (B, D, F) - cells were grown in differentiation inducing media. White circle (D) marks cells with fused/multiple nuclei after myogenic differentiation.
mass spectrometer (MS) and Masslynx® version 4.1 software (Waters Co.) in positive ion mode using data-independent acquisition (UDMSE). The capillary voltage was set at 2.8 kV, and the source temperature was set at 80 °C. Scan time was set at 0.75 s. Raw data were lock mass-corrected using the doubly charged ion of (Glu1)-fibrinopeptide B (m/z 785.8426; [M+2H]2+). Raw data files were processed and searched using ProteinLynx Global SERVER™ (PLGS) version 3.0.1 (Waters Co.). Data was analysed using trypsin as the cleavage protease, one missed cleavage was allowed, and fixed modification was set to carboxamidomethylation of cysteines, variable modification was set to oxidation of methionine. Minimum identification criteria included 1 fragment ions per peptide, 3 fragment ions and one peptide per protein. The following parameters were used to generate peak lists:

- Low energy threshold was set to 150 counts;
- Elevated energy threshold was set to 50 counts;
- Intensity threshold was set to 750 counts.

UniprotKB/SwissProt database (www.uniprot.org/) were used for protein identification. The PANTHER classification system (www.pantherdb.org/) was used for GO mapping and functional annotation of proteins. Proteins were sorted by inducible biological function and plotted in pie charts and tables describing specific protein functions.

Statistical analysis

Results were processed by using Microsoft Office Excel 2021 software (Microsoft Corporation, Redmond, Washington, USA). All proteomic results are presented as pie charts from three independent experiments (N ≥ 3 samples per group).

RESULTS

PMSCs characterisation

In order to characterise the extracted PMSCs, they were differentiated in the directions which mainly describes MSC multipotency - osteogenic, myogenic and adipogenic. It was observed that osteogenic differentiation induction induces PMSC accumulate calcium phosphate deposits in their ECM (Figure 1B). Myogenic differentiation analysis revealed that PMSCs are capable to form multinucleated cells as well (Figure 1D). Finally, adipogenic differentiation results showed lipid droplets on the cell monolayer, which is the characteristic of adipocytes (Figure 1F).

Proteomic analysis of PMSCs secretome

Further, the detail proteomic analysis of PMSCs secretomes was performed. A total of 146 proteins were detected (detail protein list is depicted in Appendix 1). Among them 55 (38%) were determined as ECM proteins and 91 (62%) were assigned as proteins belonging to other cellular regions (cellular proteins) (Figure 2A).

All determined proteins were further analysed in PHANTER classification system to determine their GO biological function. Results showed that proteins in PMSCs secretome contributes to biological processes associated with osteogenesis, cell-ECM interaction, chondrogenesis, cytoskeleton, differentiation, ECM formation, immune response, metabolism, migration, neurogenesis, signalling, transport, wound healing, and angiogenesis (Figure 2B).

Figure 2. In periosteum-derived mesenchymal stem cells secretome determined proteins grouped by their cellular localization (A) and biological functions (B).
To understand better how PMSCs secretome can stimulate osteo-regeneration process, the detail analysis of proteins, which were linked to immune response, osteo, angio, and neurogenesis biological functions, was performed. The resulting protein composition indicates the ability to promote bone regeneration to fully mature bone. The proteins found induce osteogenesis, angiogenesis and immune response. Many of them also have a synergistic effect that promotes the activities of other processes mentioned above. Specific proteins and their function in theses biological processes are listed in Tables 1 - 4.

**DISCUSSION**

Bones fractures especially in maxillofacial area or face asymmetries caused by traumas, violence, cancer excisions or other surgical treatment are exceptional because, unlike disorders in other areas, this condition is accompanied by psychosocial changes in a person’s quality of life [27]. Due to the non-physiological asymmetries and face proportions, sudden changes in the appearance of the face, a person will experience stress, because of this it is necessary to reconstruct facial tissues and return patients’ fulfilling life and self-confidence. Depending on the size of the population in the countries, facial fractures occur from a few hundred to several thousand cases per year [28]. Thus, skin, bone, cartilage reconstruction procedures are a daily clinical practice in most of the hospitals. However, widely used treatment methods still have shortcomings and this field of medicine is in the ongoing search stage for new and modern tissue regeneration techniques. Many ways have been tried to promote or accelerate better tissues healing but still there is no gold standard or reliable method in clinical practice. Stem cells have been thought to be the future of regenerative medicine, but due to poor and unstable clinical results and the existing regulation of bioethical organizations, this field of regenerative medicine is becoming less relevant [29]. Other methodologies such as blood concentrates or growth factors, have been used in clinical practice, however, it did not find a wide audience due to bioethical deficiency or small and doubtful clinical effect [10]. In recent years, cell free therapy may seem appropriate for more accurate and faster regeneration of many tissues but still needs to be better investigated in various aspects [17-19,30,31].

Table 1. Osteogenesis-inducing proteins

| No. | Gene       | Protein                          | Biological function                                                                 |
|-----|------------|----------------------------------|-------------------------------------------------------------------------------------|
| 1.  | ACTN4*     | Actinin alpha 4                  | Alpha actinin is an actin-binding protein with multiple roles in different cell types. In nonmuscle cells, the cytoskeletal isoform is found along microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane. |
| 2.  | ANXA2*     | Annexin A2                       | Calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids.                                      |
| 3.  | COL1A2*    | Collagen alpha-(f) chain         | This protein has several biological functions and bone mineralization, collagen fibril organization, odontogenesis are several of them.                 |
| 4.  | PRDX1*     | Peroxiredoxin-1                  | Protein catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols. Plays a role in cell protection against oxidative stress by detoxifying peroxides and as sensor of hydrogen peroxide-mediated signaling events. |
| 5.  | COL12A1*   | Collagen alpha-1(XII) chain      | Type XII collagen interacts with type I collagen-containing fibrils, the COL1 domain could be associated with the surface of the fibrils, and the COL2 and NC3 domains may be localized in the perifibrillar matrix. |
| 6.  | CHI3L1*    | Chitinase-3-like protein 1       | Carbohydrate-binding lectin with a preference for chitin. Play a role in tissue remodelling and in the capacity of cells to respond to and cope with changes in their environment. |
| 7.  | FBN1*      | Fibrillin-1                      | Structural component of the 10-12 nm diameter microfibrils of the extracellular matrix, which conveys both structural and regulatory properties to load-bearing connective tissues. |
| 8.  | CSF1       | Macrophage colony-stimulating factor 1 receptor | This protein plays an important role in the regulation of osteoclast proliferation and differentiation, the regulation of bone resorption, and is required for normal bone and tooth development. |
| 9.  | LRPI*      | Prolow-density lipoprotein receptor-related protein 1 | Protein is involved in regulation of actin cytoskeleton organization, regulation of extracellular matrix disassembly, positive regulation of cytosolic calcium ion concentration. |
| 10. | FBN2*      | Fibrillin-2                      | Fibrillins are structural components of extracellular calcium-binding microfibrils. Fibrillin-2-containing microfibrils regulate the early process of elastic fiber assembly. Regulates osteoblast maturation. |
| 11. | CTSK*      | Cathepsin K                      | Protein is involved in osteoclastic bone resorption and may participate partially in the disorder of bone remodelling. Displays potent endoprotease activity against fibrinogen at acid pH. Play an important role in extracellular matrix degradation. |

*Proteins cells are also involved in other biological processes.
**Table 2. Angiogenesis - inducing proteins**

| No. | Gene   | Protein                                | Biological function                                                                                                                                 |
|-----|--------|----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| 1.  | ANXA1* | Annexin A1                             | This protein plays a role in cellular response to vascular endothelial growth factor stimulus.                                                          |
| 2.  | THBS1* | Thrombospondin-1                       | This protein induce blood vessel endothelial cell migration, has a positive regulation of fibroblast migration, positive regulation of angiogenesis, positive regulation of cell population proliferation. |
| 3.  | CALR*  | Calreticulin                           | This protein has a positive regulation of endothelial cell migration.                                                                                  |
| 4.  | COL3A1*| Collagen alpha-1(III) chain            | It acts in collagen fibril organization, wound healing, cell-matrix adhesion.                                                                               |
| 5.  | HSPG2* | Basement membrane-specific heparan sulfate proteoglycan core protein | Integral component of basement membranes. Component of the glomerular basement membrane (GBM), responsible for the fixed negative electrostatic membrane charge, and which provides a barrier which is both size- and charge-selective. It serves as an attachment substrate for cells. Plays essential roles in vascularization. |
| 6.  | FBN1*  | Fibrillin-1                            | Fibrillin-1-containing microfibrils provide long-term force bearing structural support. In tissues such as the lung, blood vessels and skin, microfibrils form the periphery of the elastic fiber, acting as a scaffold for the deposition of elastin. |
| 7.  | COL4A1*| Collagen alpha-1(IV) chain             | Type IV collagen is the major structural component of glomerular basement membranes, forming a 'chicken-wire' meshwork together with laminins, proteoglycans and entactin/niidogen. |
| 8.  | SERPINE1*| Plasminogen activator inhibitor 1      | It is required for stimulation of keratinocyte migration during cutaneous injury repair.                                                             |
| 9.  | CST3*  | Cystatin-C                             | Cystatin acts as a regulator of tissue remodeling, has a negative sense to regulation of collagen catabolic process and regulation of blood vessel remodeling. |
| 10. | DPP4*  | Dipeptidyl peptidase 4                 | In association with FAP is involved in the pericellular proteolysis of the extracellular matrix (ECM), the migration and invasion of endothelial cells into the ECM. |
| 11. | KRT1*  | Keratin, type II cytoskeletal 1        | This protein plays a role in keratinization and regulation of angiogenesis.                                                                        |
| 12. | CHI3L1*| Chitinase-3-like protein 1             | Carbohydrate-binding lectin with a preference for chitin. Has no chitinase activity. Play a role in tissue remodeling and in the capacity of cells to respond to and cope with changes in their environment. |
| 13. | LRP1*  | Prollo-dense lipoprotein receptor-related protein 1 | Act as an inducer of vascular associated smooth muscle cell migration.                                                                                  |
| 14. | MMP2*  | 72 kDa type IV collagenase             | Ubiquitinous metalloproteinase that is involved in diverse functions such as remodeling of the vasculature, angiogenesis, tissue repair, tumor invasion, inflammation, and atherosclerotic plaque rupture. |
| 15. | GPNMB* | Transmembrane glycoprotein NMB         | It has a sense in regulation of angiogenesis, regulation of tissue remodeling.                                                                        |
| 16. | NRP1*  | Neuropilin-1                           | Cell-surface receptor involved in the development of the cardiovascular system, in angiogenesis, in the formation of certain neuronal circuits and in organogenesis outside the nervous system. |
| 17. | SERPIN1| Plasma protease C1 inhibitor           | Activation of the C1 complex is under control of the C1-inhibitor. It forms a proteolytically inactive stoichiometric complex with the C1r or C1s proteases. May play a potentially crucial role in regulating important physiological pathways including complement activation, blood coagulation, fibrinolysis and the generation of kinins. |

*Proteins cells are also involved in other biological processes.

**Table 3. Neurogenesis - inducing proteins**

| No. | Gene | Protein       | Biological function                                                                                   |
|-----|------|---------------|-------------------------------------------------------------------------------------------------------|
| 1.  | CALR*| Calreticulin  | This protein is involved in regulation of meiotic nuclear division, regulation of transcription, DNA-templated, protein localization to nucleus, protein export from nucleus, has a positive influence to regulation of dendritic cell chemotaxis. |
| 2.  | COL3A1*| Collagen alpha-1(III) chain | Involved in regulation of cortical development. Is the major ligand of ADGRG1 in the developing brain and binding to ADGRG1 inhibits neuronal migration and activates the RhoA pathway by coupling ADGRG1 to GNA13 and possibly GNA12. |
| 3.  | COL4A1*| Collagen alpha-1(IV) chain | This protein is involved in brain development, neuromuscular junction development.                    |
| 4.  | LRP1*| Prollo-dense lipoprotein receptor-related protein 1 | Modulate cellular events, such as APP metabolism, kinase-dependent intracellular-signalizing, neuronal calcium signaling as well as neurotransmission. |
| 5.  | S100A6| Protein S100-A6 | This protein has a function as calcium sensor and modulator, contributing to cellular calcium signaling. Plays a role in axonogenesis and signal transduction. |
| 6.  | SERPINE2*| Glia-derived nexin | Serine protease inhibitor with activity toward thrombin, trypsin, and urokinase. Promotes neurite extension by inhibiting thrombin. Binds heparin. |

*Proteins cells are also involved in other biological processes.

http://www.ejomr.org/JOMR/archives/2021/2/e2/v12n2e2ht.htm  J Oral Maxillofac Res 2021 (Apr-Jun) | vol. 12 | No 2 | e2 | p.7 (page number not for citation purposes)
| No | Gene                     | Protein                                        | Biological function                                                                                                                                                                                                 |
|----|--------------------------|------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1  | CHI3L1*                  | Chitinase-3-like protein 1                      | Plays a role in T-helper cell type 2 (Th2) inflammatory response and IL-13-induced inflammation, regulating allergen sensitization, inflammatory cell apoptosis, dendritic cell accumulation and M2 macrophage differentiation.                              |
| 2  | CD109*                   | CD109 antigen                                  | Modulates negatively TGF-beta1 signaling in keratinocytes.                                                                                                                                                               |
| 3  | SERPINE1*                | Plasminogen activator inhibitor 1              | This protein has a role in positive regulation of interleukin-8 production, positive regulation of monocyte chemotaxis, positive regulation of inflammatory response.                                                      |
| 4  | PSAP*                    | Prosecretin                                    | This protein has a sense in regulation of autophagy, platelet degranulation, neutrophil degranulation.                                                                                                               |
| 5  | ANXA2*                   | Annexin A2                                     | This annexin has a positive regulation of vacuole organization, positive regulation of vesicle fusion, vesicle budding from membrane.                                                                                  |
| 6  | ANXA5                    | Annexin A5                                     | This protein is an anticoagulant protein that acts as an indirect inhibitor of the thromboplatin-specific complex, which is involved in the blood coagulation cascade.                                    |
| 7  | FTL                      | Ferritin light chain                           | Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation. Also plays a role in delivery of iron to cells. |
| 8  | FT1H                     | Ferritin heavy chain                           | Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Ferrisodase activity. Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation.                          |
| 9  | ANXA1*                   | Annexin A1                                     | Promotes chemotaxis of granulocytes and monocytes via activation of the formyl peptide receptors. Contributes to the adaptive immune response by enhancing signaling cascades that are triggered by T-cell activation, regulates differentiation and proliferation of activated T-cells. |
| 10 | ANXA4                    | Annexin A4                                     | Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis.                                                                                                                      |
| 11 | NHR3C*                   | NTL repeat-containing protein 3                | This protein has a role in neutrophil degranulation, proteasome-mediated ubiquitin-dependent protein catabolic process, protein polyubiquitination.                                                                  |
| 12 | LGALS3BHP                | Galectin-3-binding protein                     | Promotes integrin-mediated cell adhesion. May stimulate host defense against viruses and tumour cells.                                                                                                                                 |
| 13 | DPP4*                    | Dipetidyl peptidase 4                          | Cell surface glycoprotein receptor involved in the eosinophilic signal essential for T-cell receptor (TCR)-mediated T-cell activation.                                                                                                                  |
| 14 | PTX3                     | Pentraxin-related protein PTX3                 | Plays a role in the regulation of innate resistance to pathogens, inflammatory reactions, possibly clearance of self-components and female fertility.                                                                          |
| 15 | HSPG2*                   | Basement-membrane-specific heparan sulfate glycan core protein | Endorepellin in an anti-angiogenic and anti-tumour peptide that inhibits endothelial cell migration, collagen-induced endothelial tubule morphogenesis and blood vessel growth in the chorioallantoic membrane.               |
| 16 | KRT1*                    | Keratin, type II cytoskeletal 1                | May regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITBIT1) and the receptor of activated protein C kinase 1 (RACK1). In complex with C1QBP is a high affinity receptor for kinogenous-1/HMWK. |
| 17 | GNS*                     | N-acetylglucosamine-6-sulfatase                | This protein plays a role in glycosaminoglycan catabolic process, keratin sulfate catabolic process, neutrophil degranulation.                                                                                         |
| 18 | CIR                      | Complement C1r subcomponent                    | C1r B chain is a serine protease that combines with C1q and C1s to form C1, the first component of the classical pathway of the complement system.                                                                     |
| 19 | CALR*                    | Calreticulin                                   | Calcium-binding chaperone that promotes folding, oligomeric assembly and quality control in the endoplasmic reticulum (ER) via the calreticulin/calnexin cycle. This lectin interacts transiently with almost all of the monoglycosylated glycoproteins that are synthesized in the ER. |
| 20 | CTSD*                    | Cathepsin D                                   | Acid protease active in intracellular protein breakdown. Plays a role in APP processing following cleavage and activation by ADAM30 which leads to APP degradation.                                                     |
| 21 | CTSS*                    | Cathepsin S                                   | Thiol protease. Key protease responsible for the removal of the invariant chain from MHC class II molecules and MHC class II antigen presentation.                                                                     |
| 22 | A2M*                     | Alpha-2-macroglobulin                          | This protein has a role in platelet degranulation, negative regulation of complement activation, lectin pathway.                                                                                                      |
| 23 | C4A*                     | Complement C4-A                                | Non-enzymatic component of C3 and C5 convertases and thus essential for the propagation of the classical complement pathway. Covalently binds to immunoglobulins and immune complexes and enhances the solubilization of immune aggregates and the clearance of IC through CR1 on erythrocytes. |
| 24 | LAMPI*                   | Lysosome-associated membrane glycoprotein 1    | Presents carbohydrate ligands to selectins. Also implicated in tumour cell metastasis. Acts as a receptor for Lassa virus protein.                                                                                           |
| 25 | S100A11*                 | Protein S100-A11                               | Facilitates the differentiation and the corollation of keratinocytes.                                                                                                                                                   |
| 26 | CTSB*                    | Cathepsin B                                   | Thiol protease which is believed to participate in intracellular degradation and turnover of proteins.                                                                                                                   |
| 27 | PSMA4*                   | Proteasome subunit alpha type-6                | Component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins. This complex plays numerous essential roles within the cell by associating with different regulatory particles.       |
| 28 | PDIA3*                   | Protein disulfide isomerase A3                 | This protein plays a role in protein synthesis and proteolysis of proteins.                                                                                                                                            |
| 29 | SERPINB1                 | Leukocyte elastase inhibitor                   | Neutrophil serine protease inhibitor that plays an essential role in the regulation of the innate immune response, inflammation and cellular homeostasis.                                                                     |
| 30 | SERPINH6                 | Serpin B                                      | May be involved in the regulation of serine proteases present in the brain or extraveged from the blood. Inhibitor of cathepsin G, kallikrein-8 and thrombin.                                                              |
| 31 | LGALS3                   | Galectin-3                                    | Galactose-specific lectin which binds IgE. May mediate with the alpha-3, beta-1 integrin the stimulation by CSPG4 of endothelial cells migration. Together with DMHT1, required for terminal differentiation of columnar epithelial cells during early embryogenesis. |
| 32 | PSMAT1                   | Proteasome subunit alpha type-1                | Component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins. This complex plays numerous essential roles within the cell by associating with different regulatory particles.       |
| 33 | PRDX1*                   | Peroxiredoxin-1                               | Plays a role in cell protection against oxidative stress by detoxifying peroxides and as sensor of hydrogen peroxide-mediated signaling events.                                                                           |
| 34 | SERPINH2                 | Plasminogen activator inhibitor 2             | Protein has a sense in interleukin-12-mediated signalling pathway, fibrinolysis, wound healing.                                                                                                                          |
| 35 | PPIA*                    | Peptidyl-prolyl cis-trans isomerase A          | Exerts a strong chemicotic effect on leukocytes partly through activation of one of its membrane receptors BSG/CD147, initiating a signaling cascade that culminates in MAPK/ERK activation.                                      |

*Proteins cells are also involved in other biological processes.*
Secretomes may be perfect for cell-free therapy in the treatment of gastrointestinal diseases, cancers, skin diseases, heart ischemic diseases, autoimmune disorders, can help for Covid-19 infected patients, and etc. [32-34]. However, there are very few clinical trials in this area and they have been conducted recently. Kshitiz et al. [35] used secretome of bone marrow-derived stromal cells to revealed a cardioprotective biochemical cocktail, Jarmalavičiute et al. [36] reported that secretome obtained from human dental pulp MSCs were able to reduce apoptosis of neurons. There are many successful experiments which led to further trials. Moreover, it is also known that the composition of bioactive secretome molecules depends on the type and origin of the cells [22,23,37]. In the natural environment (i.e., natural tissue) cells use these molecules to communicate with each other. By sensing each other’s expressed molecules cells know how to behave [38]. For example, in the case of bone fracture, osteoblast secreted signalling molecules composition alters. This change is further recognized by MSCs reside in surrounding tissues. These stem cells start to migrate to the damage site, were they start to differentiate into the osteoblasts. In parallel, these MSCs continue to synthesize and express their signalling molecules which is further recognized by other cells - this ensures a successful osteo-regenerative process [7,39]. Thus, as cells use the secreted molecules to communicate with each other, it is likely that cytokines, chemokines and various growth factors produced by MSCs derived from tissue close to bone should best stimulate bone regeneration. The periosteum is a tissue which is constantly connected to the bone. It performs transformation of bone tissue. As a consequence, it contains many stem cells that migrate to the site of injury in the event of bone damage and count its regeneration [40,41]. Moreover, studies have shown that PMSCs are not only multipotent (as was also confirmed by our results), but also tend to spontaneously differentiate in the osteogenic direction [17,42]. Therefore, the secretome of PMSCs should be suitable as a cell-free strategy for bone regeneration. However, the detailed protein composition and biological functions of PMSCs secretome has not been fully investigated yet. Thus, in the current study, we aimed to characterize it.

After detail proteomic analysis it was determined that PMSCs secretome is rich in proteins which is known to stimulate osteogenesis. Among the detected proteins which were related with osteogenesis, most of them were associated with bone-specific ECM formation (fibrillin-1, fibrillin-2 etc.). It is well known, that the successful MSC differentiation to osteoblasts can only occur then bone-specific ECM is forming [43]. Therefore, the initiation of such a process is important to ensure damaged bone regeneration.

During new bone formation not only osteogenesis but also angiogenesis, neurogenesis and even immune response are important [3,44]. In the beginning of bone fracture healing process, to the damage site various immune system molecules (neutrophils, monocytes, macrophages) are attracted. They not only remove necrotic cells and damage bone fragments, but also begin to secrete various inflammatory, chemotactic and progenitor mediators (e.g. stromal derived factor-1α, tumour necrosis factor α, interleukin-1β, interleukin-6, chemokine ligand 2, bone morphogenetic protein, fibroblast growth factor, WNT family proteins) in order to attract MSCs from bone marrow, periosteum or cortical bone to site of the lesion [45]. Thus, immune response stimulation is essential for the beginning of bone regeneration process. Our results showed that PMSCs secretome is also rich in various proteins which can stimulate these immune system molecules. For example, prosaposin, annexin A1, alpha-2-macroglobulin, plasminogen activator inhibitor 2, etc.

Neurogenesis is also particularly important for fully functional new bone formation. Only with the formation of a complete neural network in the bone will complete homeostasis of this tissue be ensured [46]. Proteomic analysis revealed neurogenesis-promoting proteins as follows: collagen alpha-1(IV) chain, calreticulin, etc.

Angiogenesis can be called one of the most important factors in the process of bone regeneration. During this process, different cell lines interact with each other, thus promoting tissue healing. In the formation of new bone tissue, osteogenic and angiogenic processes are closely related. The blood vessels in the bone tissue can carry minerals, growth factors, and also play a role in the physical structures around which bone deposition begins [3,4,14]. Proteomic analysis of proteins that promote angiogenesis, wound healing, and osteogenesis include thrombospondin-1, collagen alpha-1(III) chain, keratin, type II cytoskeletal 1, etc.

Finally, it is important to mention, that we have found that many proteins which were detected in PMSCs secretomes had inherent and overlapping functions. E.g., annexin A2 is involved in immune response and osteogenesis or annexin A1 is involved in angiogenesis and immune response. However, it is known from the other studies that the role of a protein may depend on the cell that synthesizes it.
And in our case, when annexins A1 and A2 are synthesized by PMSCs then their functions are to induce angiogenesis or form a new bone, but not to stimulate an immune response [20]. Furthermore, the detected proteins biological functions overlapping could also appeared because most of these proteins are not the main initiators of all those established processes [47,48]. For example, galectin-3. This protein belongs to lectin family and as known from the literature it can be involved in many different signaling pathways. It demonstrates pro-inflammatory properties by recruiting neutrophils and other immune cells to the infected sites [49]. It interacts with integrin receptors and in this way mediates cell apoptosis. It also can co-operate with various ECM proteins (collagen IV, elastin, vitronectin, etc.) and thus affect cell adhesion process [50].

CONCLUSIONS

In this study for the first time the detailed proteomic analysis of periosteum-derived mesenchymal stem cell secretome was performed. Obtained results show that cytokines, chemokines, and growth factors detected in periosteum-derived mesenchymal stem cells secretome initiates the processes required for the formation of a fully functional bone. Therefore, periosteum-derived mesenchymal stem cells secretome can be used as potential, new and innovative cell-free bone regeneration technique.

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The authors declare no conflict of interest related to this study.

REFERENCES

1. Hakeberg M, Wide Boman U. Self-reported oral and general health in relation to socioeconomic position. BMC Public Health. 2017 Jul 26;18(1):63. doi: 10.1186/s12889-017-4609-9. Erratum in: BMC Public Health. 2017 Sep 22;17(1):736. [Medline: 28741780] [PMC free article: 5530538] [doi: 10.1186/s12889-017-4609-9]

2. Masood M, Newton T, Bakri NN, Khalid T, Masood Y. The relationship between oral health and oral health related quality of life among elderly people in United Kingdom. J Dent. 2017 Jan;56:78-83. [Medline: 27825838] [doi: 10.1016/j.jdent.2016.11.002]

3. Diomede F, Marconi GD, Fonticoli L, Pizzicannella J, Merciario I, Bramanti P, Mazzon E, Trubiani O. Functional Relationship between Osteogenesis and Angiogenesis in Tissue Regeneration. Int J Mol Sci. 2020 May 3;21(9):3242. [Medline: 32375269] [PMC free article: 7247346] [doi: 10.3390/ijms21093242]

4. Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature. 2014 Mar 20;507(7492):323-328. [Medline: 24464994] [PMC free article: 4943525] [doi: 10.1038/nature13145]

5. Wang Y, Li M, Li P, Teng H, Fan D, Du W, Guo Z. Progress and Applications of Polyphosphate in Bone and Cartilage Regeneration. Biomed Res Int. 2019 Jun 27;2019:5141204. [Medline: 31346519] [PMC free article: 6620837] [doi: 10.1155/2019/5141204]

6. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Med. 2011 May 31;9:66. [Medline: 21627784] [PMC free article: 3123714] [doi: 10.1186/1741-7015-9-66]

7. Sathyendra V, Darovish M. Basic science of bone healing. Hand Clin. 2013 Nov;29(4):473-81. [Medline: 24209946] [doi: 10.1016/j.hcl.2013.08.002]

8. Katagiri W, Kawai T, Osugi M, Sugimura-Wakayama Y, Sakaguchi K, Kojima T, Kobayashi T. Angiogenesis in newly regenerated bone by secretomes of human mesenchymal stem cells. Maxillofac Plast Reconstr Surg. 2017 Mar 25;39(1):8. [Medline: 28405581] [PMC free article: 5366987] [doi: 10.1186/s40902-017-0106-4]

9. Filipowska J, Tomaszewski KA, Niedźwiedzki Ł, Walocha JA, Niedźwiedzki T. The role of vasculature in regenerated bone by secretomes of human mesenchymal stem cells. Maxillofac Plast Reconstr Surg. 2017 Mar 25;39(1):8. [Medline: 28194536] [PMC free article: 5511612] [doi: 10.1016/j.iomj.2016.07.002]

10. Daugeila P, Pranskunas M, Juodzbalys G, Lisesiene J, Banuikiotiene O, Afonso A, Sousa Gomes P. Novel cellulose/hydroxyapatite scaffolds for bone tissue regeneration: In vitro and in vivo study. J Tissue Eng Regen Med. 2018 May;12(5):1195-1208[Medline: 29498222] [doi: 10.1002/term.2651]

11. Yeo RWY, Lai RC, Tan KH, Lim SK. Exosome: A Novel and Safer Therapeutic Refinement of Mesenchymal Stem Cell. J Circc Biomark. 2013 Jan;1(1):1-12. [Medline: 33393/jcb.2013.2038]

12. Catros S, Sandgren R, Pippenger BE, Fricain JC, Herber V, El Chaar E. A Novel Xenograft Bone Substitute Supports Stable Bone Formation in Circumferential Defects Around Dental Implants in Minipigs. Int J Oral Maxillofac Implants. 2020 Nov;Dec;35(6):1122-1131. [Medline: 33270052] [doi: 10.11607/jomi.8265]

13. Zheng C, Chen J, Liu S, Jin Y. Stem cell-based bone and dental regeneration: a view of microenvironmental modulation. Int J Oral Sci. 2019 Aug 19;11(3):23. [Medline: 31423011] [PMC free article: 6802669] [doi: 10.1038/s41368-019-0060-3]

14. Takeuchi R, Katagiri W, Endo S, Kobayashi T. Exosomes from conditioned media of bone marrow-derived mesenchymal stem cells promote bone regeneration by enhancing angiogenesis. PLoS One. 2019 Nov 21;14(11):e0225472. [Medline: 31751396] [PMC free article: 6872157] [doi: 10.1371/journal.pone.0225472]
15. Robey PG. Cell sources for bone regeneration: the good, the bad, and the ugly (but promising). Tissue Eng Part B Rev. 2011 Dec;17(6):423-30. [Medline: 21797663] [PMC free article: 3223013] [doi: 10.1089/teb.2011.0199]

16. Alipour M, Nabavi SM, Arab L, Vosough M, Pakdaman H, Ehsani E, Shahpased K. Stem cell therapy in Alzheimer’s disease: possible benefits and limiting drawbacks. Mol Biol Rep. 2019 Feb;46(1):1425-1446. [Medline: 30565076] [doi: 10.1007/s10008-014-4499-7]

17. Samsonraj RM, Raghanathan M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. Stem Cells Transl Med. 2017 Dec;6(12):2173-2185. [Medline: 29076267] [PMC free article: 5702523] [doi: 10.1002/stcm.17-0129]

18. Gangadaran P, Rajendran RL, Lee HW, Kalimuthu S, Hong CM, Jeong SY, Lee SW, Lee J, Ahn BC. Extracellular vesicles from mesenchymal stem cells activates VEGF receptors and accelerates recovery of hindlimb ischemia. J Control Release. 2017 Oct 28;264:112-126. [Medline: 28837823] [doi: 10.1016/j.jconrel.2017.08.022]

19. Katagiri W, Watanabe T, Toyama N, Osugi M, Sakaguchi K, Hibi H. Clinical Study of Bone Regeneration by Conditioned Medium From Mesenchymal Stem Cells After Maxillary Sinus Floor Elevation. Dent. 2017 Aug;26(4):607-612. [Medline: 28727618] [doi: 10.1007/s12272-019-01198-x]

20. Deshmukh AS, Peisj L, Beaudry J, Jespersen NZ, Nielsen CH, Ma T, Brunner AD, Larsen TJ, Bayarri-Olmos R, Prabhakar BS, Helstrang C, Severinsen MCK, Holst B, Kjaer A, Tang-Christensen M, Sanfridson A, Garred P, Privé GG, Pedersen BK, Gerhart-Hines Z, Nielsen S, Drucker DJ, Mann M, Scheele C. Proteomics-Based Comparative Mapping of the Secretomes of Human Brown and White Adipocytes Reveals EPDR1 as a Novel Batokine. Cell Metab. 2019 Nov 5;30(5):963-975.e7. [Medline: 31688873] [doi: 10.1016/j.cmet.2019.10.001]

21. Kehd D, Generali M, Mallone A, Heller M, Uldry AC, Cheng P, Gantenbein B, Hoerstrup SP, Weber B. Proteomic analysis of human mesenchymal stem cell secretomes: a systematic comparison of the angiogenic potential. NJP Regen Med. 2019 Apr 16;4:8. [Medline: 31016031] [PMC free article: 6467904] [doi: 10.1038/s41536-019-0070-x]

22. Requicha JF, Viegas CA, Albuquerque CM, Azevedo JM, Reis RL, Gomes ME. Effect of anatomical origin and cell passage number on the stemness and osteogenic differentiation potential of canine adipose-derived stem cells. Stem Cell Res Rep. 2012 Dec;6(4):1211-22. [Medline: 22773405] [doi: 10.1007/s12015-012-9397-0]

23. Otto A, Collins-Hooper H, Patel K. The origin, molecular regulation and therapeutic potential of myogenic stem cell populations. J Anat. 2009 Nov;215(5):477-97. [Medline: 19702867] [PMC free article: 2780567] [doi: 10.1111/j.1469-7580.2009.01338.x]

24. Phetfong J, Supokawej A, Wattanapanitch M, Kheolamai P, Issaragrisil S. Cell type of origin influences iPSC generation and differentiation to cells of the hematopoietic lineage. Cell Tissue Res. 2016 Jul;365(1):101-12. [Medline: 26893154] [doi: 10.1007/s00441-016-2369-y]

25. Lee AR, Moon DK, Siregar A, Moon SY, Yeon RH, Byun JH, Woo DK. Involvement of mitochondrial biogenesis during the differentiation of human periosteum-derived mesenchymal stem cells into adipocytes, chondrocytes and osteocytes. Arch Pharm Res. 2019 Dec;42(12):1052-1062. [Medline: 31016030] [PMC free article: 27927618] [doi: 10.1007/s12015-012-9397-0]

26. Wisniewski JR, Zouman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009 May;6(5):359-62. [Medline: 19377485] [PMC free article: 2307907] [doi: 10.1038/nmeth.1322]

27. Lee KH, Chua J. Psychosocial Support Following Maxillofacial Trauma and its Impact on Trauma Recurrence. J Maxillofac Oral Surg. 2018 Mar;17(1):32-37. [Medline: 29382991] [PMC free article: 5772019] [doi: 10.1007/s12663-016-0979-2]

28. Werlinger F, Villalón M, Duarte V, Acevedo R, Aguiarera R, Alcocer D, Badillo O, Briones R, Condal C, Del Río M, García R, Herrera J, Jaramillo M, Merchán F, Nasi M, Osbén R, Rivera A, Riviello S, Rojas P, Vidal C, Rodríguez G, Schild S, Sehilt S, Arroyo E, Alvarado MJ, Sepúlveda P, Cortés J. Trends of maxillofacial trauma: An update from the prospective register of a multicenter study in emergency services of Chile. Med Oral Patol Oral Cir Bucal. 2019 Sep 1;24(5):e588-e594. [Medline: 31433390] [PMC free article: 6746707] [doi: 10.4317/medoral.22985]

29. Gramignoli R, Sallustio F, Widera D, Raschzok N. Editorial: Tissue Repair and Regenerative Mechanisms by Stem/Progenitor Cells and Their Secretome. Front Med (Lausanne). 2019 Jan 31;6:11. [Medline: 30766784] [PMC free article: 6356970] [doi: 10.3389/fmed.2019.00011]

30. Samasaekia R, Rabiee B, Putra I, Chen X, Park YJ, Hemmati P, Eslami L, Djaliani AR. Effect of Human Cornal Mesenchymal Stromal Cell-derived Exosomes on Corneal Epithelial Wound Healing. Invest Ophthalmol Vis Sci. 2018 Oct 1;59(12):5194-5200. [Medline: 30372477] [PMC free article: 6203220] [doi: 10.1167/iovs.18-24803]

31. Park SR, Kim JW, Jun HS, Roh JY, Lee HY, Hong JS. Stem Cell Secretome and Its Effect on Cellular Mechanisms Relevant to Wound Healing. Mol Ther. 2018 Feb 7;26(2):606-617. [Medline: 29066165] [PMC free article: 5835016] [doi: 10.1038/s41397-017-0293-x]

32. Baghahi K, Tokhanbigli S, Asadzadeh H, Nmaki S, Reza Zali M, Hashemi SM. Extracellular vesicles as a novel cell-free therapeutic approach in gastrointestinal diseases. J Cell Physiol. 2019 Jul;234(7):9910-9926. [Medline: 30536985] [doi: 10.1002/jcp.27934]

33. Kahmini FR, Shahgaldi S. Therapeutic potential of mesenchymal stem cell-derived extracellular vesicles as novel cell-free therapy for treatment of autoimmune disorders. Exp Mol Pathol. 2021 Feb;118:104566. [Medline: 31360961] [doi: 10.1016/j.exmpath.2020.104566]

34. Rezakhani L, Kelishadrokhi AF, Soleimanizadeh A, Rahmati S. Mesenchymal stem cell (MSC)-derived exosomes as a cell-free therapy for patients Infected with COVID-19: Real opportunities and range of promises. Chem Phys Lipids. 2021 Jan;234:105009. [Medline: 33189639] [PMC free article: 7658620] [doi: 10.1016/j.chemphyslip.2020.105009]
35. Kshitiz, Ellison DD, Suhail Y, Afzal J, Woo L, Kilic O, Spees J, Levchenko A. Dynamic secretome of bone marrow-derived stromal cells reveals a cardioprotective biochemical cocktail. Proe Natl Acad Sci U S A. 2019 Jul 9;116(28): 14374-14383. [Medline: 31239339] [PMC free article: 6628676]

36. Jarmalavičiūtė A, Tunaïtis V, Pivoraité U, Venalis A, Pivoriūnas A. Exosomes from dental pulp stem cells rescue human dopaminergic neurons from 6-hydroxy-dopamine-induced apoptosis. Cytotherapy. 2015 Jul;17(7):932-9. [Medline: 25981557] [doi: 10.1016/j.jcyt.2014.07.013]

37. Hernández-Monjaraz B, Santiago-Osorio E, Monroy-Garcia A, Ledesma-Martinez E, Mendoza-Núñez VM. Mesenchymal Stem Cells of Dental Origin for Inducing Tissue Regeneration in Periodontitis: A Mini-Review. Int J Mol Sci. 2018 Mar 22;19(4):944. [Medline: 2956801] [PMC free article: 3979585] [doi: 10.3390/jims19040944]

38. Song D, Yang D, Powell CA, Wang X. Cell-cell communication: old mystery and new opportunity. Cell Biol Toxicol. 2019 Apr;35(2):89-93. [Medline: 30815784] [doi: 10.1007/s10565-019-09470-y]

39. Lin H, Sohn J, Shen H, Langhans MT, Tuan RS. Bone marrow mesenchymal stem cells: Aging and tissue engineering applications to enhance bone healing. Biomaterials. 2019 May;203:96-110. [Medline: 29980291] [PMC free article: 6733253] [doi: 10.1016/j.biomaterials.2018.06.026]

40. Dwek JR. The perioseum: what is it, where is it, and what mimics it in its absence? Skeletal Radiol. 2010 Apr;39(4): 319-23. [Medline: 20049593] [PMC free article: 2826636] [doi: 10.1007/s00256-009-0849-9]

41. Xiaoy H, Wang L, Zhang T, Chen C, Chen H, Li S, Hu J, Hu L. Periostem progenitors could stimulate bone regeneration in aged murine bone defect model. J Cell Mol Med. 2020 Oct;24(20):12199-12210. [Medline: 32931157] [PMC free article: 7579685]

42. Allen MR, Hock JM, Burr DB. Perioseum: biology, regulation, and response to osteoporosis therapies. Bone. 2004 Nov;35(5):1003-12. [Medline: 1542024] [doi: 10.1016/j.bone.2004.07.014]

43. Singh P, YashRoy RC, Hoque M. Augmented bone-matrix formation and osteogenesis under magnetic field stimulation in vivo XRD, TEM and SEM investigations. Indian J Biochem Biophys. 2006 Jun;43(3):167-72. [Medline: 16967906]

44. Collins MT, Stratakis CA. Bone Formation, Growth, and Repair. Horm Metab Res. 2016 Nov;48(11):687-688. [Medline: 27871113] [PMC free article: 19055404-119907]

45. Schlundt C, El Khassawna T, Serra A, Dienelt A, Wendler S, Schell H, van Rooijen N, Radbruch A, Lucius R, Hartmann S, Duda GN, Schmidt-Bleek K. Macrophages in bone fracture healing: Their essential role in endochondral ossification. Bone. 2018 Jan;106:78-89. [Medline: 2659389] [PMC free article: 10.1016/j.bone.2015.10.019]

46. Liu Q, Lei L, Yu T, Jiang T, Kang Y. Effect of Brain-Derived Neurotrophic Factor on the Neurogenesis and Bone. 2018 Jan;106:78-89. [Medline: 29390590] [PMC free article: 10.1098/ten.tea.2017.0462]

47. Nooren IM, Thornton JM. Diversity of protein-protein interactions. EMBO J. 2003 Jul 15;22(14):3486-92. [Medline: 12853464] [PMC free article: 165629]

48. Ishikawa Y, Otsu K, Oshikawa J. Caveolin; different roles for insulin signal? Cell Signal. 2005 Oct;17(10):1175-82. [Medline: 15913956] [PMC free article: 10.1016/j.cellsig.2005.03.025]

49. Diaz-Alvarez L, Ortega E. The Many Roles of Galectin-3, a Multifaceted Molecule, in Innate Immune Responses against Pathogens. Mediators Inflamm. 2017;2017:9247574. [Medline: 28607536] [PMC free article: 5457773] [doi: 10.1155/2017/9247574]

50. Dwek JR. The periosteum: what is it, where is it, and what mimics it in its absence? Skeletal Radiol. 2010 Apr;39(4): 319-23. [Medline: 10.1055/s-0042-119907]

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Appendix 1A. Full list of proteins detected in PMSCs secretome

| Accession | Gene | Full protein name |
|-----------|------|-------------------|
| G1SGH0    | PRSS2| Serine protease   |
| G1TTO6    | SELENBP1| Selenium binding protein 1 |
| G1TWY4    | NAGLU| N-acetyl-alpha-glucosaminidase |
| G1TRZ2    | LAMP1| Lysosomal associated membrane protein 1 |
| G1TWX6    | GPMB| Glycoprotein mmb |
| G1SRL4    | NAGA| Alpha-galactosidase |
| P37153    | AP0D| Apolipoprotein D |
| G1SMH9    | NHLRC3| NHL repeat containing 3 |
| G1TH29    | COCH| Cochlín |
| G1NT8     | ANX6| Annexin |
| G1TDN8    | KRT73| Keratin 73 |
| G1S30     | NRP1| Neuropilin |
| G1U304    | GALNS| Uncharacterized protein |
| G1SS91    | CA4| Uncharacterized protein |
| G1TPZ1    | LGALS1| Galectin |
| Q9TT75    | NPC1| Niemann-Pick type C1 disease protein |
| G1SCT1    | PREP| Prolyl endopeptidase |
| G1SM91    | FAH| Fumarylacetoacetate hydrolase |
| A5HC63    | Serpinb2| Plasminogen activator inhibitor 2 (Fragment) |
| G1T610    | NAALAD2| N-acetylated alpha-linked acidic dipeptidase 2 |
| G1T366    | DNP| Aspartyl aminopeptidase |
| P43236    | CTSK| Cathepsin K |
| G1SQ70    | A2M| Alpha-2-macroglobulin |
| Q9TTC6    | PPIA| Peptidyl-prolyl cis-trans isomerase A |
| G1TAU6    | SERPIN1| Serpin family E member 1 |
| G1TIC1    | DPP4| Dipeptidyl peptidase 4 |
| G1STF7    | TF| Serotransferrin |
| G1SK33    | ITGB1| Integrin beta |
| G1SQK1    | SERPINB6| Serpin family B member 6 |
| G1TFU9    | LGALS3BP| Galectin 3 binding protein |
| P0939     | TPI| Triosephosphate isomerase |
| G1SFK1    | SMPDL3A| Acid sphingomyelinase-like phosphodiesterase |
| G1SU5     | FBN2| Fibrillin 2 |
| G1THB5    | PLBD1| Phospholipase B-like |
| G1SCJ8    | SERPING1| Plasma protease C1 inhibitor |
| G1TK56    | HEXB| Hexosaminidase subunit beta |
| O19053    | ADH5| Alcohol dehydrogenase class-3 |
| G1SDT0    | ACTBL2| Actin, beta like 2 |
| G1SM6     | PRDX4| Peroxiredoxin 4 |
| G1TAB2    | GM2A| Uncharacterized protein |
| G1SK9     | TXNRD1| Uncharacterized protein |
| G1TRA1    | C1QTNF5| C1q and TNF related 5 |
| G1SMA6    | LOC100348947| Alpha-mannosidase |
| G1T484    | COL4A2| Collagen type IV alpha 2 chain |
| Q7M2X2    | COL4A1| Collagen alpha 1(IV) chain (Fragment) |
| G1TR31    | LOC100353346| Uncharacterized protein |
| G1TKY9    | CTHRC1| Collagen triple helix repeat containing 1 |
| G1T2A9    | FLNC| Filamin C |
| G1SW90    | KRT25| Keratin 25 |
| G1T4Z1    | LRP1| LDL receptor related protein 1 |
| G1SPP3    | KRT4| Keratin 4 |
| G1SR53    | FUCAL| Alpha-L-fucosidase |
| P09451    | FTI| Ferritin light chain |
| G1SKF1    | THBS1| Thrombospondin 1 |
| G1SLM4    | SERPIN1| Serpin family E member 2 |
| P25915    | FTH1| Ferritin heavy chain (Fragment) |
| G1SL41    | GUSB| Beta-glucuronidase |
| G1SY34    | ENO1| Eno1ase 1 |
| G1SN67    | SERPINB1| Serpin family B member 1 |
| P51662    | ANX1| Annexin A1 |
| G1USB3    | PSAP| Prosaposin |
| G1SKM2    | FBN1| Fibrillin 1 |
| G1SUZ7    | ARSA| Arylsulfatase A |
| G1TJC6    | GAPDH| Glyceraldehyde-3-phosphate dehydrogenase |
| P14282    | COL8A1| Collagen alpha-1(VIII) chain |
| G1TN89    | HSPG2| Heparan sulfate proteoglycan 2 |
| A5HC45    | CTS| Cathepsin D (Fragment) |
| G1SL62    | ANXA2| Annexin |

http://www.ejomr.org/JOMR/archives/2021/2/e2/v12n2e2ht.htm  |  J Oral Maxillofac Res 2021 (Apr-Jun) | vol. 12 | No 2 | e2 | p.13
(page number not for citation purposes)
Appendix 1B. Full list of proteins detected in PMSCs secretome

| Accession | Gene | Full protein name |
|-----------|------|-------------------|
| P13019    | BLMH | Bleomycin hydrolase (Fragment) |
| G1SD9     | COL6A2 | Collagen type VI alpha 2 chain |
| G1SF9     | PAM | Peptidylglycine alpha-amidating monoxygenase |
| G1TE7     | KRT20 | Uncharacterized protein |
| B7NZ49    | NPM1 | Nucleophosmin 1 isoform 1 (Predicted) |
| G1SR15    | CD109 | CD109 molecule |
| G1SPX1    | FBLN2 | Fibulin 2 |
| G1SJX3    | CP | Ceruloplasmin |
| G1U8S7    | CSF1 | Colony stimulating factor 1 |
| G1SUH8    | KRT2 | Keratin 2 |
| G1TY7     | KRT14 | Keratin 14 |
| G1SIK0    | SERPIN1 | Serpin family C member 1 |
| G1T994    | COL12A1 | Collagen alpha-1(XII) chain |
| G1J6N3    | NPC2 | NPC intracellular cholesterol transporter 2 |
| G1T1V0    | KRT10 | Keratin 10 |
| G1TDN6    | KRT5 | Keratin 5 |
| G1U758    | KRT18 | Uncharacterized protein |
| P10658    | PSAF1 | Phosphoserine aminotransferase |
| G17T8A    | CPQ | Carboxypeptidase Q |
| G1SW59    | VIM | Vimentin |
| O97529    | ANX8 | Annexin A8 |
| G1SQ9V9   | LOC100352842 | Uncharacterized protein |
| G1T9M9    | HSPA8 | Heat shock protein family A (Hsp70) member 8 |
| G1SQ02    | PRDX1 | Peroxiredoxin 1 |
| G1SA8     | PSMA1 | Proteasome endopeptidase complex |
| G1U9T4    | NME2 | Nucleoside diphosphate kinase |
| G1TM41    | LAMC1 | Uncharacterized protein |
| G1SI29    | LOC100359245 | Thioredoxin |
| P62975    | UBB | Ubiquitin |
| G11A83    | ANXA4 | Annexin |
| G1TBY1    | CTSB | Cathepsin B |
| G1U9H8    | KRT1 | Keratin 1 |
| O97862    | CST3 | Cystatin-C |
| G1SKE3    | LOC100351904 | Uncharacterized protein |
| G1SF6     | GP1 | Glucose-6-phosphate isomerase |
| G1SW70    | HEXA | Beta-hexosaminidase |
| P49065    | ALB | Serum albumin |
| G1TE6     | ANX5 | Annexin |
| G1T9V4    | PSMA6 | Proteasome subunit alpha type |
| G1TJP4    | CHI3L1 | Chitinase 3 like 1 |
| Q29426    | KRT3 | Keratin, type II cytoskeletal 3 |
| G1ISQ0    | GNS | N-acetylglucosamine-6-sulfatase |
| G1SHZ4    | KRT7 | Keratin 7 |
| G1U9R6    | FN1 | Fibronectin |
| P47845    | LGALS3 | Galectin-3 |
| G1SV24    | LOC100000830 | Uncharacterized protein |
| G1SEG6    | LOXL1 | Lysyl oxidase like 1 |
| P24480    | S100A11 | Protein S100-A11 |
| G1SW24    | AARS | Alanyl-tRNA synthetase |
| G1TIQ2    | CALM1 | Uncharacterized protein |
| G1SVY8    | CKB | Creatine kinase B-type |
| P13943    | MMP1 | Interstitial collagenase |
| G1SLG2    | CD248 | CD248 molecule |
| P30891    | S100A6 | Protein S100-A6 |
| G1TQR0    | ACTN1 | Actinin alpha 1 |
| P13491    | LDHA | L-lactate dehydrogenase A chain |
| G1TUC8    | ACTN4 | Actinin alpha 4 |
| P15253    | CALR | Calreticulin |
| G1SQG5    | MDH1 | Malate dehydrogenase |
| G1SYN4    | PTX3 | Pentraxin 3 |
| P41975    | SOD3 | Extracellular superoxide dismutase [Cu-Zn] |
| P50757    | MMP2 | 72 kDa type IV collagenase |
| Q9TRZ7    | TIMP2 | Metalloproteinase inhibitor 2 |
| G1U9R8    | GS | Gelsolin |
| G1SVK5    | S100A4 | Protein S100 |
| G1TOK7    | CTSS | Cathepsin S |
| G1T8J0    | COL3A1 | Collagen type III alpha 1 chain |
| G1SRW4    | EMILIN1 | Elastin microfibril interlacer 1 |
### Appendix 1C. Full list of proteins detected in PMSCs secretome

| Accession | Gene      | Full protein name                          |
|-----------|-----------|--------------------------------------------|
| G1T7H9    | C1R       | Complement C1r                             |
| G1TAH7    | TKT       | Transketolase                              |
| G1T2Z5    | COL1A2    | Collagen alpha-2(I) chain                 |
| P18287    | APOE      | Apolipoprotein E                           |
| G1SQE2    | USP36     | Uncharacterized protein                    |
| G1TYA7    | LDHB      | L-lactate dehydrogenase                    |
| G1T8S8    | LOC100346772 | Alpha-mannosidase                 |
| B7NZF1    | PDIA3     | Protein disulphide-isomerase              |
| G1TC10    | LOC100347623 | Uncharacterized protein            |
| G1SX17    | TCN2      | Transcobalamin 2                          |
| G1SR14    | DNAH12    | Uncharacterized protein                    |
| G1SYM4    | A1BG      | Alpha-1B-glycoprotein                      |
| G1SN83    | LAMB2     | Lammin subunit beta 2                     |
| G1SDP2    | FKBP10    | FK506-binding protein                     |
| G1SMV1    | MVP       | Major vault protein                       |
| U3KJN6    | LMNA      | Lamin A/C                                 |
| G1TC42    | AXL       | AXL receptor tyrosine kinase              |
| G1SNd0    | QSOX1     | Sulfhydrol oxidase                        |
| G1SE6I    | FLNB      | Filamin-B                                 |
| G1SJD6    | VCL       | Vinculin                                  |
| B7NZD7    | PSMB4     | Proteasome subunit beta                   |
| G1SXN3    | LAMA4     | Lammin subunit alpha 4                    |
| P21195    | P4HB      | Protein disulphide-isomerase              |
| G1SU71    | PSMB1     | Proteasome subunit beta                   |
| G1U7Y3    | LOXL3     | Lysyl oxidase like 3                      |
| Q5ENG7    | TIMP-1    | Tissue inhibitor of metalloproteinase 1 (Fragment) |
| G1SL68    | MYP4      | Myosin heavy chain 9                      |
| G1SKT3    | ANGPTL2   | Angiopoietin like 2                       |
| G1SPR5    | GLB1      | Uncharacterized protein                    |
| G1T4W4    | FBLN5     | Fibulin 5                                 |
| G1SN21    | PNP       | Purine nucleoside phosphorylase           |
| G1SIY5    | CTBS      | Chitobiase                                |
| G1T9Y0    | NTR4      | Netrin 4                                  |
| P30946    | HSP90AA1  | Heat shock protein HSP 90-alpha           |
| D5K340    | APOE      | Apolipoprotein E4 (Fragment)             |
| G1TAV9    | TGFB3     | Transforming growth factor beta receptor 3 |
| G1SJD0    | PTK7      | Protein tyrosine kinase 7 (inactive)      |
| G1U1Q1    | THBS2     | Thrombospindin 2                          |
| G1T670    | SORT1     | Proteasome subunit alpha type             |
| G1SMB4    | ADAMTS1L1 | ADAMTS like 1                             |
| G1S560    | CFH       | Uncharacterized protein                    |
| G1S340    | LOX       | Lysyl oxidase                             |
| G1SHP2    | STC1      | Stanniocalcin 1                           |
| G1TB95    | SVEP1     | Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1 |
| G1T7Q2    | LOXL2     | Lysyl oxidase like 2                      |
| G1SDZ0    | CTS4      | Carboxypeptidase                          |
| G1TBS8    | CFH       | Uncharacterized protein                    |
| G1SKC5    | SERPINF1  | Serpin family F member 1                  |
| G1SN22    | FSTL1     | Follistatin like 1                        |
| G1TBP5    | IL1RL1    | Interleukin 1 receptor like 1             |
| U3KPG6    | ICAM1     | Intercellular adhesion molecule 1         |
| G1TB96    | COL15A1   | Collagen type XV alpha 1 chain            |
| G1T7U6    | EFEMP1    | EGF containing fibulin extracellular matrix protein 1 |
| G1U3U2    | CLSTN1    | Calsytentin 1                             |
| G1SER8    | PFN1      | Profilin                                  |
| G1THR3    | CTSS      | Cathepsin Z                               |
| G1TY46    | ISLR      | Immunoglobulin superfamily containing leucine rich repeat |
| G1T8D7    | AEBP1     | AE binding protein 1                      |
| G1T7T1    | UGP2      | UTP--glucose-1-phosphate uridyltransferase |
| G1UA0A4   | BGN       | Biglycan                                  |
| P01885    | B2M       | Beta-2-microglobulin                      |
| G1SP97    | LUM       | Lumican                                   |
| G1T7G7    | C1S       | Complement C1s                            |
| G1U4C2    | CCDC80    | Coiled-coil domain containing 80          |
| Q6GVI2    | COL1A1    | Prepro-alpha-1 collagen type I (Fragment) |
| G1T8L2    | COL5A2    | Collagen type V alpha 2 chain             |
| P28663    | MMP3      | Stromelysin-1                            |
| G1TM9     | SPARC     | SPARC                                     |
| Q09XSC5   | CLU       | Clusterin                                 |