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. After characterisation PMSCs secretomes were collected and their proteomic analysis was performed. 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 periosteum. 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 acepromazine (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

articainum/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 scizors tissue was minced into smaller pieces, approximately 1 mm^3 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 filtrating 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 cm^2 Falcon flasks, at the density of 40,000 cells/cm^2. 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% CO_2. 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 µM dexamethasone (Sigma-Aldrich Co.), 1 µM indomethacin (Sigma-Aldrich Co.; Darmstadt, Germany), 500 µM 3-isobutyl-1-methylxanthine and 10 µg/ml human recombinant insulin. Cells were grown in this media for 4 days. Then, cells were fixed with 4% formaldehyde for 10 min at room temperature. Latter fixed cells were washed with distilled water followed by 5 min washing with 60% isopropanol at room temperature. Lipid reserves stored in the cells were stained with Oil Red O staining solution (0.5% solution in isopropanol - Sigma-Aldrich Co, Darmstadt, Germany) for 10 to 15 min. After removing the dye, cells were washed with deionized water until the water becomes clear. Red stained droplets were visualized and captured with a charge-coupled device (CCD) camera (EXi Blue™ - QImaging; Surrey, British Columbia, Canada) attached to inverted phase contrast microscope (Olympus IX51 - Olympus Co.; Tokyo, Japan). For myogenic differentiation induction, cells were grown in DMEM supplemented with 2% horse serum and 1% penicillin streptomycin solution, for one week. Cell differentiation was evaluated by visualizing multinuclear cells with Crystal violet staining. Briefly, myogenic differentiation media was removed, cells 3 times washed with PBS, and stained with 0.1% Crystal violet solution for 30 min (prepared in 20% ethanol; Sigma-Aldrich Co.). Then cells were washed with distilled water and multinuclear cells 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. Differentiation was carried out for 21 days, half of the media was replaced every two/three days. 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/cm^2 in the 75 cm^2 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% CO_2 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.

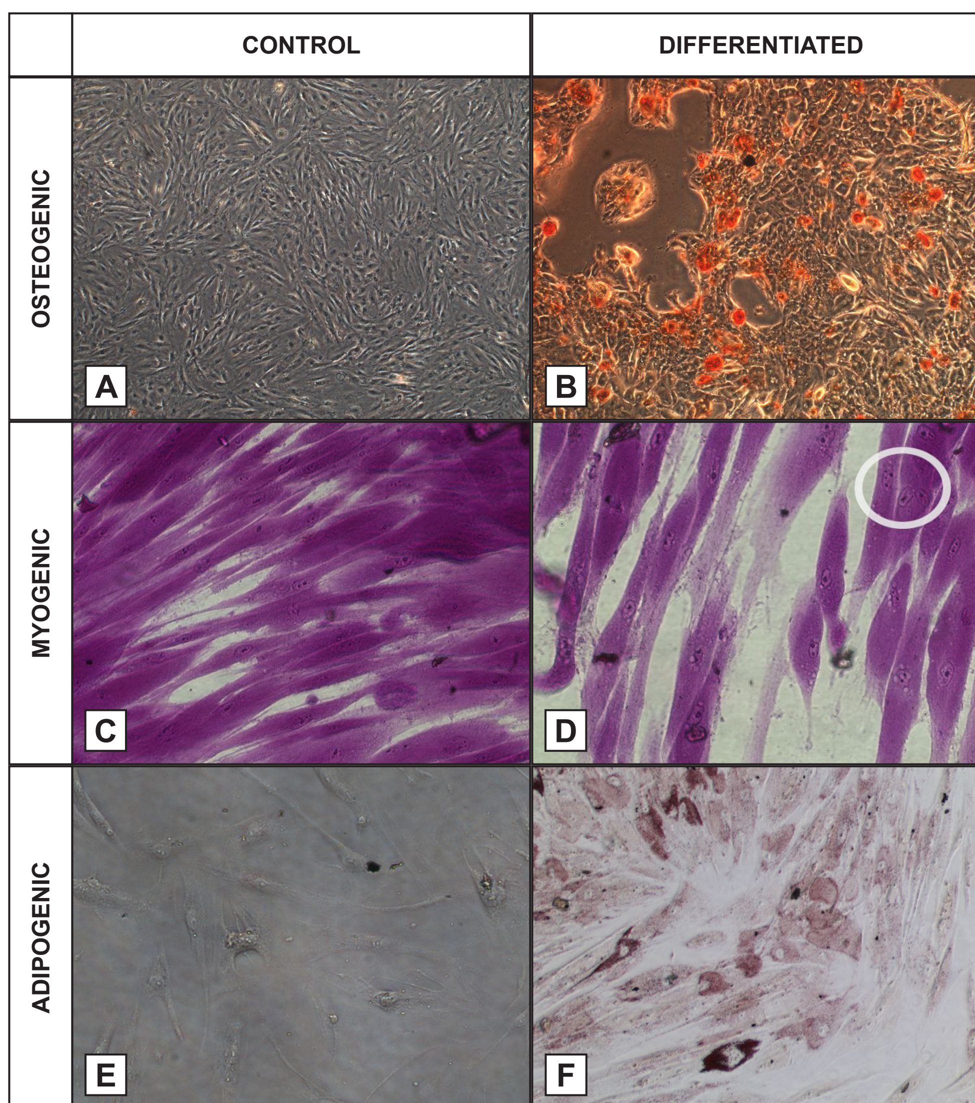

Figure 1. Evaluation of periosteum-derived mesenchymal stem cells differentiation potential: A, B = osteogenic differentiation lineage (original magnification x10); C, D = myogenic differentiation lineage (original magnification x40); adipogenic differentiation lineage (original magnification x40). Control 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.

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 NH_4HCO_3 pH 8.0. Protein digestion was then performed by adding trypsin in 50 µL of 50 mM NH_4HCO_3 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% CH_3CN. The eluates were combined, acidified with 10% CF_3COOH 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

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 carbamidomethylation 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).

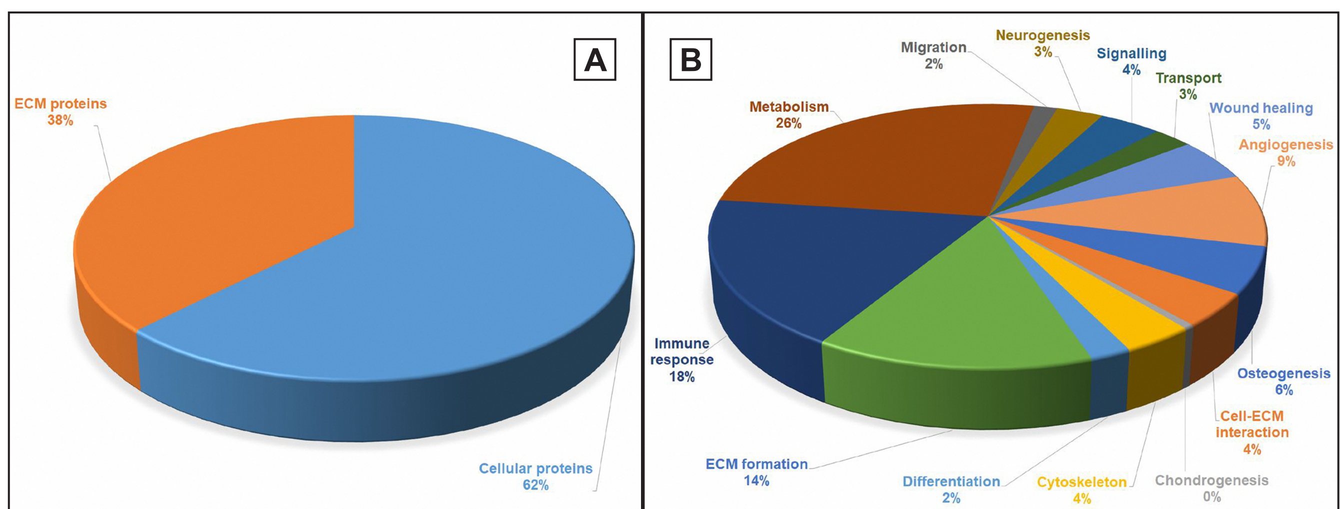

Figure 2. In periosteum-derived mesenchymal stem cells secretome determined proteins grouped by their cellular localization (A) and biological functions (B).

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-2(I) 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.	LRP1*	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/nidogen.
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*	Prolow-density 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.	SERPING1	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*	Prolow-density lipoprotein receptor-related protein 1	Modulate cellular events, such as APP metabolism, kinase-dependent intracellular signaling, 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.

Table 4. Proteins that has a sense in immune response

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 TGFB1 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*	Prosaposin	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 thromboplastin-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.	FTH1	Ferritin heavy chain	Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Has ferroxidase 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.	NHLRC3*	NHL repeat-containing protein 3	This protein has a role in neutrophil degranulation, proteasome-mediated ubiquitin-dependent protein catabolic process, protein polyubiquitination.
12.	LGALS3BP	Galectin-3-binding protein	Promotes integrin-mediated cell adhesion. May stimulate host defense against viruses and tumour cells.
13.	DPP4*	Dipeptidyl peptidase 4	Cell surface glycoprotein receptor involved in the costimulatory 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 proteoglycan core protein	Endorepellin in an anti-angiogenic and anti-tumour peptide that inhibits endothelial cell migration, collagen-induced endothelial tube 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 (ITB1) and the receptor of activated protein C kinase 1 (RACK1). In complex with C1QBP is a high affinity receptor for kininogen-1/HMWK.
17.	GNS*	N-acetylglucosamine-6-sulfatase	This protein plays a role in glycosaminoglycan catabolic process, keratan sulfate catabolic process, neutrophil degranulation.
18.	C1R	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 monoglucosylated 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.	LAMP1*	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 cornification of keratinocytes.
26.	CTSB*	Cathepsin B	Thiol protease which is believed to participate in intracellular degradation and turnover of proteins.
27.	PSMA6*	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 antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent, positive regulation of extrinsic apoptotic signalling pathway.
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.	SERPINB6	Serpin B6	May be involved in the regulation of serine proteinases present in the brain or extravasated 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 DMBT1, required for terminal differentiation of columnar epithelial cells during early embryogenesis.
32.	PSMA1*	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.	SERPINB2	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 chemotactic 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 leaded 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 tissue 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, chemotaxic 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.

Neurogensis 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.

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

Accession	Gene	Full protein name
G1SGH0	PRSS2	Serine protease
G1TT06	SELENBP1	Selenium binding protein 1
G1TWY4	NAGLU	N-acetyl-alpha-glucosaminidase
G1TRZ2	LAMP1	Lysosomal associated membrane protein 1
G1TXW6	GPNMB	Glycoprotein nmb
G1SRL4	NAGA	Alpha-galactosidase
P37153	APOD	Apolipoprotein D
G1SMH9	NHLRC3	NHL repeat containing 3
G1T2H9	COCH	Cochlin
G1SNT8	ANXA6	Annexin
G1TDN8	KRT73	Keratin 73
G1SM30	NRP1	Neuropilin
G1U304	GALNS	Uncharacterized protein
G1SS91	C4A	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)
G1T6T0	NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2
G1T3E6	DNPEP	Aspartyl aminopeptidase
P43236	CTSK	Cathepsin K
G1SQ70	A2M	Alpha-2-macroglobulin
Q9TTC6	PPIA	Peptidyl-prolyl cis-trans isomerase A
G1TAU6	SERPINE1	Serpin family E member 1
G1T1C1	DPP4	Dipeptidyl peptidase 4
G1STF7	TF	Serotransferrin
G1SK33	ITGB1	Integrin beta
G1SQK1	SERPINB6	Serpin family B member 6
G1TFU9	LGALS3BP	Galectin 3 binding protein
P00939	TPI1	Triosephosphate isomerase
G1SFK1	SMPDL3A	Acid sphingomyelinase-like phosphodiesterase
G1SUS5	FBN2	Fibrillin 2
G1THB5	PLBD1	Phospholipase B-like
G1SCJ8	SERPING1	Plasma protease C1 inhibitor
G1TKS6	HEXB	Hexosaminidase subunit beta
O19053	ADH5	Alcohol dehydrogenase class-3
G1SDT0	ACTBL2	Actin, beta like 2
G1SMI6	PRDX4	Peroxiredoxin 4
G1TAB2	GM2A	Uncharacterized protein
G1SKS9	TXNRD1	Uncharacterized protein
G1TRA1	C1QTNF5	C1q and TNF related 5
G1SMA6	LOC100348947	Alpha-mannosidase
G1T548	COL4A2	Collagen type IV alpha 2 chain
Q7M2X2	Col4a1	Collagen alpha 1(IV) chain (Fragment)
G1TR31	LOC100353846	Uncharacterized protein
G1TKY9	CTHRC1	Collagen triple helix repeat containing 1
G1T2A9	FLNC	Filamin C
G1SWB0	KRT25	Keratin 25
G1T4Z1	LRP1	LDL receptor related protein 1
G1SPP3	KRT4	Keratin 4
G1SR53	FUCA1	Alpha-L-fucosidase
P09451	FTL	Ferritin light chain
G1SKF1	THBS1	Thrombospondin 1
G1SLM4	SERPINE2	Serpin family E member 2
P25915	FTH1	Ferritin heavy chain (Fragment)
G1SL41	GUSB	Beta-glucuronidase
G1SYJ4	ENO1	Enolase 1
G1SN67	SERPINB1	Serpin family B member 1
P51662	ANXA1	Annexin A1
G1U5B3	PSAP	Prosaposin
G1SKM2	FBN1	Fibrillin 1
G1SUZ7	ARSA	Arylsulfatase A
G1TJG6	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase
P14282	COL8A1	Collagen alpha-1(VIII) chain
G1TN89	HSPG2	Heparan sulfate proteoglycan 2
A5HC45	CTSD	Cathepsin D (Fragment)
G1SL62	ANXA2	Annexin

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

Accession	Gene	Full protein name
P13019	BLMH	Bleomycin hydrolase (Fragment)
G1SD89	COL6A2	Collagen type VI alpha 2 chain
G1SFR5	PAM	Peptidylglycine alpha-amidating monooxygenase
G1TE79	KRT20	Uncharacterized protein
B7NZF9	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
G1T1Y7	KRT14	Keratin 14
G1SIK0	SERPINC1	Serpin family C member 1
G1T994	COL12A1	Collagen alpha-1(XII) chain
G1T6N3	NPC2	NPC intracellular cholesterol transporter 2
G1T1V0	KRT10	Keratin 10
G1TDN6	KRT5	Keratin 5
G1U758	KRT18	Uncharacterized protein
P10658	PSAT1	Phosphoserine aminotransferase
G1T7A8	CPQ	Carboxypeptidase Q
G1SWS9	VIM	Vimentin
O97529	ANXA8	Annexin A8
G1SQV9	LOC100352842	Uncharacterized protein
G1T9M9	HSPA8	Heat shock protein family A (Hsp70) member 8
G1SQ02	PRDX1	Peroxiredoxin 1
G1SDA8	PSMA1	Proteasome endopeptidase complex
G1U9T4	NME2	Nucleoside diphosphate kinase
G1T4M1	LAMC1	Uncharacterized protein
G1SJZ9	LOC100359245	Thioredoxin
P62975	UBB	Ubiquitin
G1TA83	ANXA4	Annexin
G1TBY1	CTSB	Cathepsin B
G1U9I8	KRT1	Keratin 1
O97862	CST3	Cystatin-C
G1SKE3	LOC100351904	Uncharacterized protein
G1SFG6	GPI	Glucose-6-phosphate isomerase
G1SWR0	HEXA	Beta-hexosaminidase
P49065	ALB	Serum albumin
G1TED6	ANXA5	Annexin
G1T9V4	PSMA6	Proteasome subunit alpha type
G1TJP4	CHI3L1	Chitinase 3 like 1
Q29426	KRT3	Keratin, type II cytoskeletal 3
G1SQU0	GNS	N-acetylglucosamine-6-sulfatase
G1SHZ4	KRT7	Keratin 7
G1U9R6	FN1	Fibronectin
P47845	LGALS3	Galectin-3
G1SV24	LOC100008830	Uncharacterized protein
G1SEG6	LOXL1	Lysyl oxidase like 1
P24480	S100A11	Protein S100-A11
G1SW24	AARS	Alanyl-tRNA synthetase
G1T1Q2	CALM1	Uncharacterized protein
G1SVY8	CKB	Creatine kinase B-type
P13943	MMP1	Interstitial collagenase
G1SLG2	CD248	CD248 molecule
P30801	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	GSN	Gelsolin
G1SVK5	S100A4	Protein S100
G1T0K7	CTSS	Cathepsin S
G1T8J0	COL3A1	Collagen type III alpha 1 chain
G1SRW4	EMILIN1	Elastin microfibril interfacer 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
G1SQC2	USP36	Uncharacterized protein
G1TYA7	LDHB	L-lactate dehydrogenase
G1T8S8	LOC100346772	Alpha-mannosidase
B7NZF1	PDIA3	Protein disulfide-isomerase
G1TCT0	LOC100347623	Uncharacterized protein
G1SX17	TCN2	Transcobalamin 2
G1SRT4	DNAH12	Uncharacterized protein
G1SYM4	A1BG	Alpha-1B-glycoprotein
G1SN83	LAMB2	Laminin subunit beta 2
G1SDP2	FKBP10	FK506-binding protein
G1SVM1	MVP	Major vault protein
U3KNJ6	LMNA	Lamin A/C
G1TC42	AXL	AXL receptor tyrosine kinase
G1SND0	QSOX1	Sulfhydryl oxidase
G1SE61	FLNB	Filamin-B
G1SJ56	VCL	Vinculin
B7NZD7	PSMB4	Proteasome subunit beta
G1SXN3	LAMA4	Laminin subunit alpha 4
P21195	P4HB	Protein disulfide-isomerase
G1SU71	PSMB1	Proteasome subunit beta
G1U7Y3	LOXL3	Lysyl oxidase like 3
Q5ENG7	TIMP-1	Tissue inhibitor of metalloproteinase 1 (Fragment)
G1SL68	MYH9	Myosin heavy chain 9
G1STK3	ANGPTL2	Angiopoietin like 2
G1SPR5	GLB1	Uncharacterized protein
G1T4W4	FBLN5	Fibulin 5
G1SN21	PNP	Purine nucleoside phosphorylase
G1SIY5	CTBS	Chitobiase
G1T9Y0	NTN4	Netrin 4
P30946	HSP90AA1	Heat shock protein HSP 90-alpha
D5G340	APOE	Apolipoprotein E4 (Fragment)
G1TAV9	TGFBR3	Transforming growth factor beta receptor 3
G1SIJ0	PTK7	Protein tyrosine kinase 7 (inactive)
G1U1Q1	THBS2	Thrombospondin 2
G1T670	SORT1	Proteasome subunit alpha type
G1SMR4	ADAMTSL1	ADAMTS like 1
G1SS69	CFB	Uncharacterized protein
G1SJG0	LOX	Lysyl oxidase
G1SPH2	STC1	Stanniocalcin 1
G1TB95	SVEP1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1
G1T7Q2	LOXL2	Lysyl oxidase like 2
G1SDZ0	CTSA	Carboxypeptidase
G1TBS8	CFH	Uncharacterized protein
G1SCK5	SERPINF1	Serpin family F member 1
G1SNI2	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	Calsyntenin 1
G1SER8	PFN1	Profilin
G1TRH3	CTSZ	Cathepsin Z
G1TY46	ISLR	Immunoglobulin superfamily containing leucine rich repeat
G1T8D7	AEBP1	AE binding protein 1
G1TI71	UGP2	UTP--glucose-1-phosphate uridylyltransferase
G1U0A4	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
P28863	MMP3	Stromelysin-1
G1T2M9	SPARC	SPARC
Q9XSC5	CLU	Clusterin