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Original article

Proteomic analysis of hypoxia and non-hypoxia secretome mesenchymal stem-like cells from human breastmilk

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ABSTRACT

Introduction: Breastmilk contains proteins and cells which have stem cell properties. The human breastmilk stem cell mimick mesenchymal stem cells and expresses pluripotency genes. The protein level of breastmilk is high in colostrum and gradually subsides in the first year of lactation. The mesenchymal stem cells from breastmilk can be an alternative source of stem cells that can potentially affect cardiovascular therapy. This study aimed to identify the proteomic analysis of secretome mesenchymal stem-like cells under hypoxia compared to non-hypoxia from human breastmilk stem cells.

Material and methods: The human breastmilk was collected from six healthy breastfeeding women and transported to the laboratory under aseptic conditions. The breastmilk cells were isolated then cultured. After 72 h, the human breastmilk stem cells reached confluence then cleaned up and isolated in serum-free media (spheroid) to allow serial passaging every 48 h. The acquisition stem cell was made with flow cytometry. The cells were divided into hBSC secretomes under hypoxia (A) and non-hypoxia (B) and analyzed for LC-MS to identify the peptide structure.

Results: The human breastmilk cells contained several mesenchymal stem-like cells in density 2.4×10^6 cell/mL for hypoxia and 2×10^6 cell/mL for non-hypoxia conditions. The human breastmilk stem cell surface markers derived from the third cell passage process were 93.77% for CD44, 98.69% for CD73, 88.45% for CD90, and 96.30% for CD105. The protein level of secretome mesenchymal stem -like cells under hypoxia was measured at 5.56 μg/mL and 4.28 μg/mL for non-hypoxia. The liquid chromatography-mass spectrometry analysis identified 130 and 59 peptides from hypoxia and non-hypoxia of the human breastmilk stem cell secretome sequentially. Some important proteomics structures were found in the hypoxic human breastmilk stem cell secretome, such as transforming growth factor-β, VE-cadherin, and caspase.

Conclusion: The human breastmilk cells contain mesenchymal stem-like cells and a high concentration of CD44, CD73, CD90, and CD105 as surface markers at third passage culture. The hypoxic hBSC secretome produces a higher protein level compare to non-hypoxia. The transforming growth factor -β was found in the hypoxic hBSC secretome as a modulator of VEGF-mediated angiogenesis.

Abbreviations: AFP, Alpha-Fetoprotein; ATP, Adenosine Triphosphate; BD, Becton Dickinson; BMPR-II, Bone morphogenetic protein type II; BSA, Bovine Serum Albumin; cDNA, complementary Deoxyribonucleic Acid; EHD3, EH Domain-containing Protein 3; FACS, Fluorescence-Activated Cell Sorting; FBS, Fetal Bovine Serum; hBSC, Human Breastmilk Stem Cell; HIF-1α, Hypoxia Inducible Factor-1α; IGF1, Insulin-like Growth Factor 1; LALBA, α-Lactalbumin; LC-MS, Liquid Chromatography-Mass Spectrometry; LF, Lactoferrin; MAPK, Mitogen-Activated Protein Kinase; MPZL1, Myelin Protein Zero-like Protein 1; MPS, Multi Proliferative Supplement; mRNA, messenger Ribonucleic Acid; MSC, Mesenchymal Stem Cell; PBS, Phosphate-buffered Saline; SDS, Sodium Dodecyl Sulfate; SMA, Smooth Muscle Actin; TGF-β, Transforming Growth Factor-Beta; VEGF, Vascular Endothelial Growth Factor; SMAD, Signals Mothers Against the Decapentaplegic.

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1. Introduction

Breastmilk contains functionally bioactive components, including protein and growth factors. Human milk comprises approximately 98% luminal and myoepithelial cells under healthy conditions (Hassiotou et al., 2012) (Ninkina et al., 2019). Milk composition can be varied among women, populations and developed during evolution to fulfill newborn and infants need (Ballard and Morrow, 2013). The protein level of breastmilk is high in colostrum. Colostrum is produced in small amounts in the first few days after delivery, rich in immunological components such as secretory IgA, lactoferrin, leukocytes, and epidermal growth factors. (Lyons et al., 2020) . During the first week of lactation, the protein concentration is high and gradually decreases in the first year (early: 14–16 g/L, 3–4 months: 8–10 g/L, 6 months: 7–8 g/L, and gradually declining) (Haschke et al., 2017). Breastmilk cells are divided into blood-derived and breast-derived cells, and a few progenitor/stem cells are known as part of mammary stem cells (Hassiotou et al., 2012) . Breast milk stem cells/progenitor colonies were positively identified and stained for the mammary stem cell marker CK5+ and the general stem cell marker Nestin (Cregan et al., 2007) (Hassiotou et al., 2012)(released the first report demonstrating pluripotency markers expression by cell sub-populations in breastmilk, named human breastmilk stem cells (hBSC). The human breastmilk stem cells consist of lactoferrin, cytokine, hormone, and growth factor that can potentially affect immunomodulator, inflammatory process, angiogenesis, and tissue regeneration (Twigger et al., 2015).

The International Society for Cellular Therapy suggested that mesenchymal stem cell (MSC) in breastmilk can be identified based on markers to express by MSC (STRO-1, CD90 (Thy-1), CD105 (endoglin), and CD73), and the ability of breastmilk-derived cells to differentiate into mesodermal cells (osteoblasts, chondrocytes, and adipocytes), a known feature of MSC-like cells (Díez et al., 2015)(Ullah et al., 2015). (Tang et al. (2019) found breastmilk cells positive for MSC-like cells marker CD44, CD73, CD90, and CD105.

Many studies have been previously conducted in breastmilk stem cells but lack standardization of the methodology to isolate, process, and identify breastmilk (Via et al., 2012). This study aimed to identify the difference of human breastmilk stem cells (hBSC) secretomes by proteomic analysis that mimics mesenchymal stem cells under hypoxia and non-hypoxia states.

2. Materials and methods

2.1. Breastmilk sample collection

Six healthy breastfeedings women, lactation period under one month, were recruited and volunteer in this study after collecting informed consent. The subjects donated 50 mL breastmilk using a manual breast pump and assembled in the morning and processed within 3 h after collection. They were transported to the laboratory immediately under aseptic conditions. This experiment was registered and approved by the Ethical Committee of Universitas Sebelas Maret, Surakarta, Indonesia (No. 353/UN27.06/KEPK/EC/2019).

2.2. Breastmilk cell isolation

Breastmilk was mixed and diluted 1:1 with Phosphate-buffered saline (PBS) and 2% Penicillin/Streptomycin in 50 mL conical tube and centrifuged at 3000 rpm for 20 min at 20 °C. After centrifugation, the fat layer and skimmed liquid were discarded, and the cell pellet was resuspended in 2 mL PBS, then transferred into 15 mL conical tube. The cell pellet was re-centrifuged at 3000 rpm for 10 min and added with 2 mL PBS after removing the supernatant. We repeated the centrifugation process for 10 min at 3000 rpm.

2.3. Culture of human breastmilk stem cell

Breastmilk cells were cultured with Mammocult (STEMCELL ™ Technologies, Canada) and incubated at 37 °C and 21% O_2, 5% CO_2, for 72 h. MammoCult™ consisted of 3 mL basal media and was added with Multi Proliferative Supplement (MPS) consist of 10% Human Proliferation Factor, 20% Fetal Bovine Serum (FBS), 2% Penicillin/Streptomycin, 0.5% Amphotericin B, Heparin (6 μg/mL), and Hydrocortisone 15 μL for first passage. After the third day, the cells were diluted with PBS and digested with Trypsin to clean up serum peptide.

2.4. Fluorescence-Activated cell Sorting (FACS)

The mesenchymal stem cells were isolated and characterized using Fluorescence-Activated Cell Sorting (FACS) with markers CD44, CD73, CD90, and CD105 cultured in a spheroid medium. All antibodies were bought from Becton Dickinson (BD) company. The hBSC culture was incubated in a dark room for 15 min at room temperature, then washed by PBS, and 1 mL of FBS was added before being centrifuged at 1000 rpm for 10 min resuspended with PBS. The data were analyzed by CellQuest software.

2.5. Culture of mesenchymal stem-like cells in spheroid media

The stem cell cultured for second passage using Mammocult™ basal media serum-free (spheroid media) and change the media every 48 h. After the third passage on the 7th day, stem cells were divided into hypoxia and non-hypoxia. Hypoxia intervention was made using a hypoxia chamber containing 1% O_2, 10% CO_2 incubated at 37 °C for 72 h and then examined using an Inverted NIKON Eclipse Tt 2000-U microscope with objective lens magnification of 4–8–10 to identify the viable cell. Only the live cells would be considering for MSC-like cell identification.

2.6. Secretome of mesenchymal stem-like cells isolation

Breastmilk stem cells in spheroid media from hypoxia and non-hypoxia treatment are accommodated and centrifuged at 3000 rpm for 3 min at 4 °C. Stem cells are centrifuged back at 1500 rpm for 5 min at 4 °C. The final supernatant under hypoxia and non-hypoxia condition in the form of breastmilk stem cell secretion isolates in the spheroid medium can then be stored at −80 °C for proteomic analysis.

2.7. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The human breastmilk-like stem cells secretome was divided into secretomes under hypoxia (A) and non-hypoxia (B). Methods used for this study include protein concentration determination and then in-solution digestion preparation sample. The procedure for protein concentration was determined using the Bradford method according to the User Guide Coomassie (Bradford) Protein Assay KIT protocol. Data were analyzed by Thermo SkanIt RE software for Multiskan Go Software version 3.2. The sample was then prepared for in-solution digestion examination by adding solution digestion before LC-MS analysis. The preparation involved reduction, alkylation, and digestion. Ten μg of protein was designed to split the protein into peptides using the in-solution digestion

method according to User Guide Thermo in Solution and Guanidination KIT. The Liquid Chromatography-Mass Spectrometry analysis was conducted by UHPLC Vanquish Tandem Q-Exactive Plus Orbitrap HRMS ThermoScientific. Peptides (injection volume 5 μL) were directly injected onto an Accucore Phenyl Hexyl 100 × 2.1 mm (ThermoScientific, 2.6 μm) column. Peptides were resolved over a gradient from 0% Acetonitrile and 20% water, 0.1% formic acid (E) to 2% E over 3 min, 2% to 35% E over 27 min, 35% to 90% E over 15 min, and conversely, 90% to 5% E over 1 min with a flow rate 0.3 mL/min at 50 °C. The mass spectrometry (MS) analysis was carried out on a Q-Exactive HF mass spectrometer (ThermoScientific) using HCD fragmentation in positive mode. The control was made using Bovine Serum Albumin (BSA) peptide to ensure the preparation method was running well. The mass spectrometer scans were acquired from 200 to 2000 *m/z*. The data were analyzed using Proteome Discoverer 2.2 software with Sequest HT as a search engine. Peptide and protein level identification were both set to a false discovery rate (strict) of 1% and false discovery rate (relaxed) of 5% using a Percolator strategy, and proteins must have score sequence HT > 0 and unique peptide ≥ 2. Mass tolerance was set to 10 ppm. Enzyme specificity was set to Trypsin (cleavages C-terminal to Lys and Arg) with a maximum of 2 missed cleavages permitted. Dynamic modification was set to Acetyl / +59.011 Da (N-Terminus), Oxidation / +15.995 Da (M) and static modification was set to Carbamidomethyl / +57.021 Da (C).

3. Results

3.1. Mesenchymal stem-like cells identification of breastmilk

The breastmilk cells were cultured in Mammocult (STEMCELL™ Technologies). The viable cell showed a spindle-shaped microscope mimic the mesenchymal stem cell (MSC) characteristic in density 2.4 × 10^6 cell/mL under hypoxia condition and 2 × 10^6 cell/mL non-hypoxia. The cells are shown in Fig. 1. Subsequently, we executed flow cytometric characterization of various cell surface MSC markers (CD73, CD90, CD105) and myoepithelial cells (CD44) in breastmilk. The markers that showed MSC-like cell properties in breastmilk-isolated cells (marker CD44, CD73, CD90, and CD105) are shown in Fig. 2.

The data showed different results between MSC-like cells from the first and third cell passaging processes. Positive markers for MSC-like cells from first cell passaging processes were relatively lower 50.75% for CD44, 79% for CD73, 10.23% for CD90, 45.74% for CD105 than MSC-like cells from third cell passaging processes were 93.77% for CD44, 98.69% for CD73, 88.45% for CD90, 96.30% for CD105. The mesenchymal stem-like cell indicator at the first and third passage is shown in Fig. 3.

3.2. Protein level examination of human breastmilk stem cells hypoxia compare to non-hypoxia

The protein levels examination of MSC-like cells using the Bradford method is shown in Figs. 4 and 5. The highest protein level was 5.56 μg/ml for sample A (hypoxia). Sample A had 32 peaks peptides (the highest peak was 34.22), and sample B (non-hypoxia) had 33 peaks peptides (the highest peak was 34.16). The same height was found from both samples, 17.65, 24.43, and 29.84. Each peak was compared to the MS spectrum based on the UniProtKB database. Among 130 proteins identified from sample A and 59 proteins identified from sample B, we found a total of 7 uncharacterized proteins from both samples. Furthermore, we detected 17 identical proteins and sorted the proteins from samples A and B. The protein type identification of samples A and B were shown in Table 1.

4. Discussion

4.1. The human breastmilk stem cell as mesenchymal stem-like cells

In this study, multi-proliferative supplement (MPS) and 20% Fetal Bovine Serum (FBS) supplementations in Mammocult (STEMCELL™ Technologies) on the first passage have promoted stem cell growth. The FBS provides growth factors, adhesion factors, and vital nutrients essential for the cells (Díez et al., 2015) (Manley, 2013) Stem cell replication was optimized by spheroid-conditioned media with basal media serum-free. The uncontrollable variable could affect experimental results. (The previous studies have found the MSC-like cells in breastmilk-isolated cells (Abd Allah et al., 2016, (Hosseini et al., 2014) (Ninkina et al., 2019) (Patki et al., 2010) (Pichiri et al., 2016) (Tang et al., 2019) has strengthened the present study, where the cells were positive for MSC markers, analyzed with flow cytometry procedure (CD44, CD73, CD90, and CD105).

Positive markers for MSC-like cells from the first cell passaging process were relatively lower than MSC-like cells from the third cell passaging process. The high level in the third passage because of the purification cell process under passages. The first cell passaging process was inadequate to produce distilled cells. Therefore, we only obtained a low percentage of all MSC markers (<90%). The higher percentage results were obtained from more pure cells acquired from third cell passaging process. Multiple cell passaging processes were suggested to get purified MSC (Tang et al., 2019) (Tang et al., 2019). The mesenchymal stem cell proliferation is influenced by media conditions, including nutritional level, cell confluence, oxygen level, number of passages, and plastic surface quality (Ullah et al., 2015) (Via et al., 2012)(Vizoso et al., 2017) The in vitro cell culture underwent an aging process, which

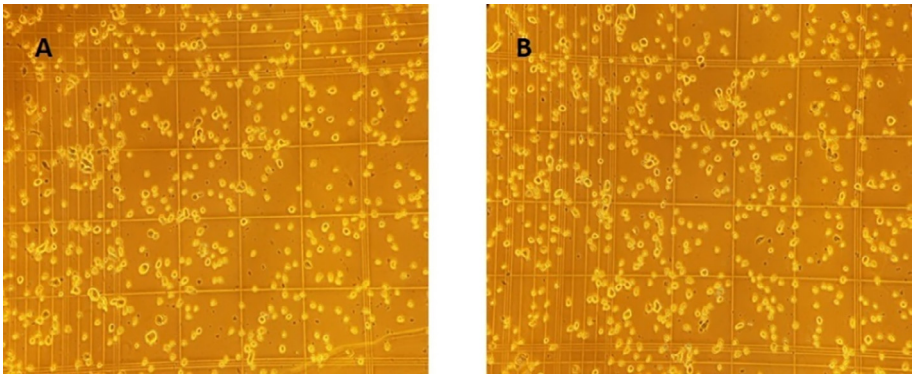

Fig. 1. Identification and cell count of human breastmilk stem cell (hBSC) in Neubauer chamber. A) The hBSC under hypoxia, B) The hBSC non-hypoxia.

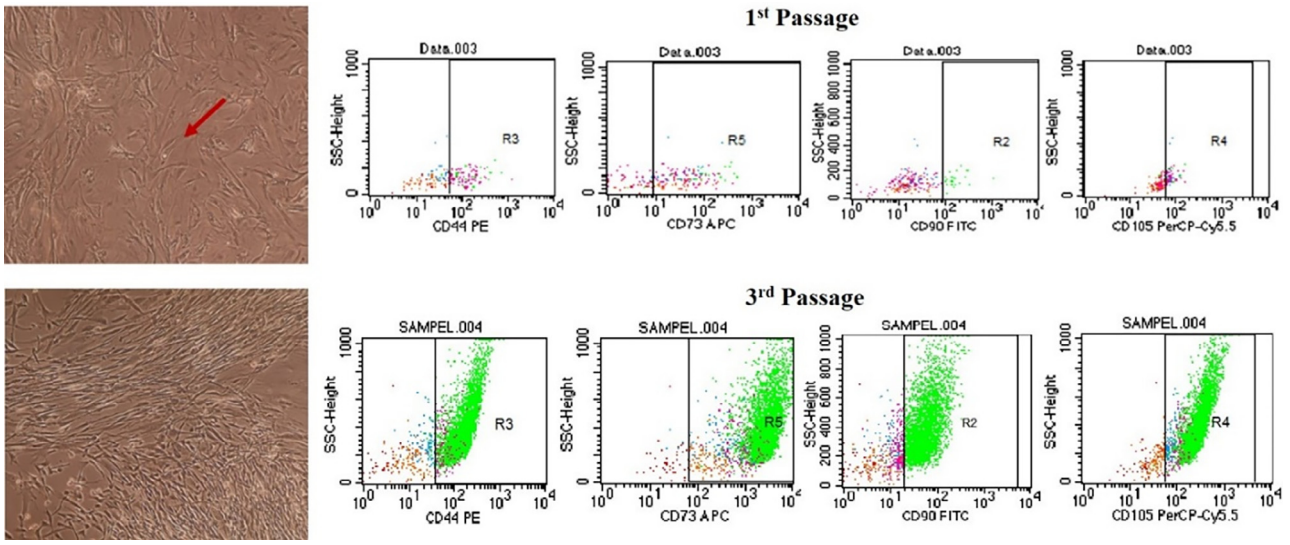

Fig. 2. Morphologies and identification of CD-44, CD-73, CD-90, and CD-105 mesenchymal stem cell surface markers at first and third passages of hBSC by flow cytometry. Representative MSC-like cells (as shown by the arrow).

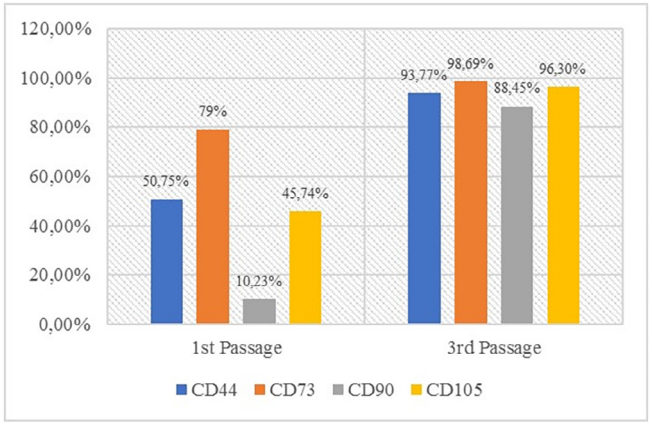

Fig. 3. The mesenchymal stem-like cells indicator at first and third passage.

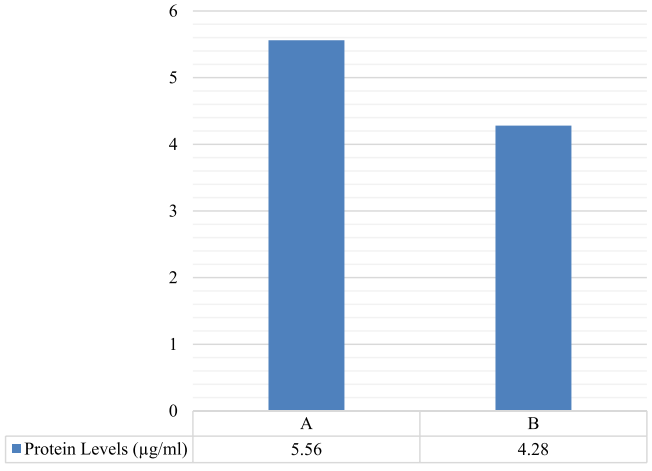

Fig. 4. The protein levels of secretome mesenchymal stem-like cells from human breastmilk. (A) hypoxia, (B) non-hypoxia.

changed the cell's morphology, and decreased the proliferative capacity (Via et al., 2012). The decrease in telomerase activity is one reason behind the aging process (Ullah et al., 2015) Vacanti et al. (2005) investigated passage numbers and their effect on MSC characteristics and reported that late MSC (>15 passages) had limited differentiation. However, early MSC (<5 passages) remained pluripotent (Sancricca, 2010). Like the number of stem cells, the innate breastmilk sample, phenotype, and the pluripotency expression also play an essential role (Briere et al., 2016). Breastmilk sample and age donor's physicochemical properties may alter the MSC proliferation ability (Ninkina et al., 2019). The low density and expression of the cells might cause poor MSC results (Tang et al., 2019). These particular factors may define the low result of CD90 (<90%) despite its multiple passaging processes in this study.

4.2. Hypoxic intervention on human breastmilk-stem cells in spheroid media

After third passage in spheroid-conditioned media, human breastmilk stem cells (hBSC) incubated in a hypoxic chamber using 1% O_2, 10% CO_2 at 37 °C for 72 h. Hypoxia condition activates Hypoxia Inducible Factor (HIF-1α) in transmembrane cellular through P38-MAPK (mitogen-activated protein kinase) pathway to release bone morphogenetic protein (BMP) (Wang et al., 2015). The BMP binds to the bone morphogenetic protein receptor (BMPR) to activate the signals through mothers against the decapentaplegic (SMAD) complex to stimulate proliferation and inhibit apoptosis (Guo et al., 2012) by promoting more hBSC. In the present experiment, the hypoxia condition successfully promotes replication of hBSC more than non-hypoxia. The density of hypoxia hBSC in the study was 2.4×10^6 cell/mL, while non-hypoxia showed 2×10^6 cell/mL in the confluence harvest period. It looked similar proliferation rate on both groups, but hypoxia secretome hBSC has the highest protein determination level.

4.3. The protein level in mesenchymal stem-like cells from breastmilk-isolated cells

The protein level in breastmilk would gradually decrease with lactation progression and vary among donors, while the storage and handling of breastmilk affect the composition (Haschke et al., 2016). The isolation procedure plays a vital role in detaching milk compounds and purifying milk-specific proteins (Van Herwijnen et al., 2016). Culture medium and supplements, culture

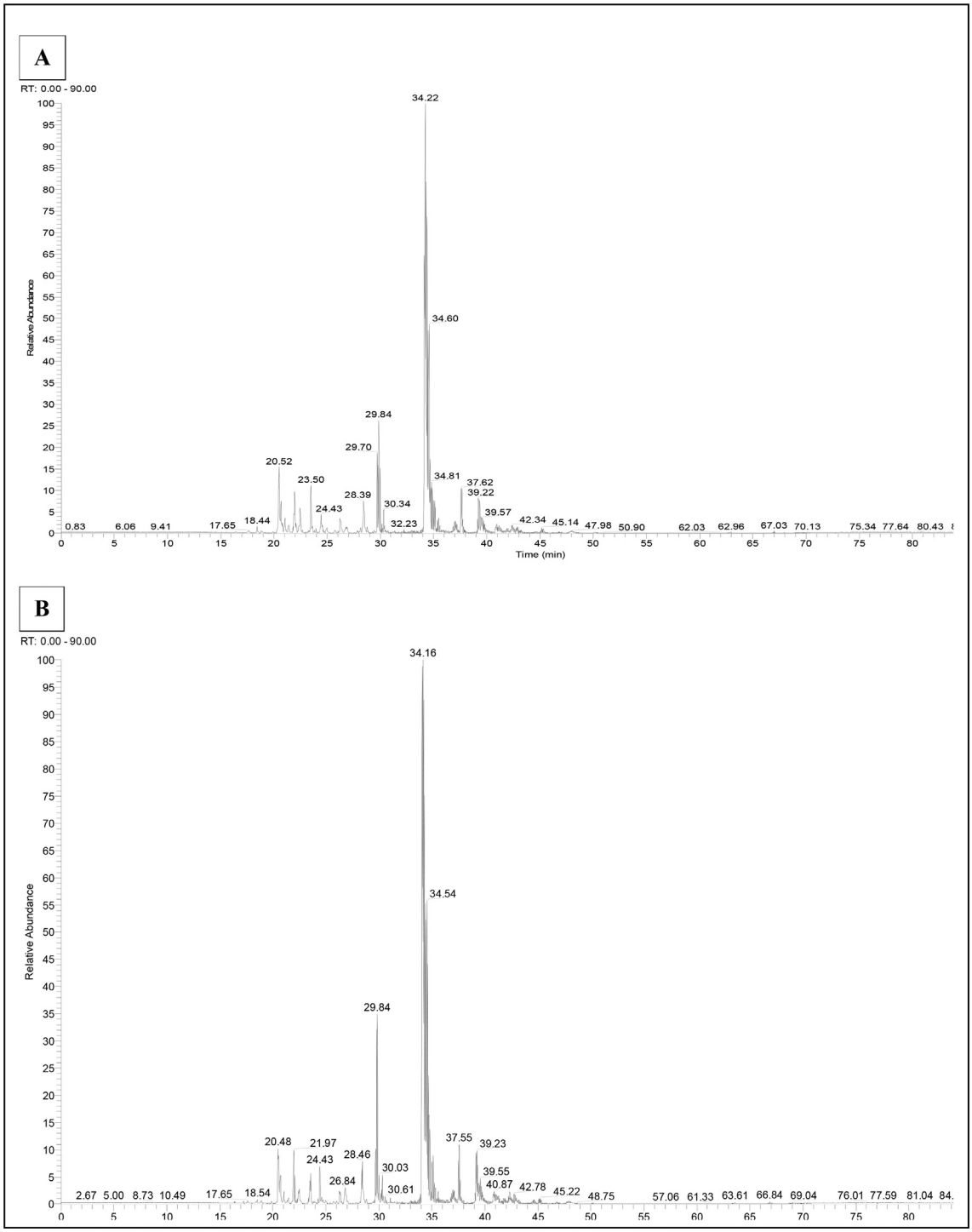

Fig. 5. Relative abundance and retention time (RT) of protein in human breastmilk stem cell secretome by Liquid Chromatography-Mass Spectrometry. A) Hypoxia, B) non-hypoxia.

duration, and conditions can modify the innate secretome (Vizoso et al., 2017) The amount of peptide from breastmilk cells secretome under hypoxia conditions was varied, including actin-myosin epidermal cells, collagen, mRNA, TGF-β, VE-Cadherin, Caspase, IGF1, Alpha-Fetoprotein (AFP), and LF. TGF-β act as a controller of cellular responses and homeostasis of endothelium vascular. The binding of TGF-β initiates TGF-β signaling to its serine/threonine receptors through ligand binding, activation of SMAD proteins through phosphorylation, regulation of target genes expression in association with DNA-binding partners, and regulation of SMAD activity and degradation (Kubiczkova et al., 2012). The Bone morphogenetic protein type II receptor (BMPR-II) is a receptor belonging to the TGF-β superfamily. The BMP receptors typically activate SMAD1/5/8 via BMPR-II, whereas TGF-β receptors activate SMAD2/3 via the ALK5 (Upton and Morrell, 2013). The SMAD2/3 or SMAD1/5/8 are phosphorylated and coalescence with SMAD4, which translocates to the nucleus and modulates transcription of target genes (Maarman et al., 2013). The mesenchymal stem cells will use low oxygen concentration in vivo to proliferate, differentiate, and regenerate (Sancricca, 2010) (Vizoso

Table 1
The protein identification of human breastmilk stem cell secretome, hypoxia (sample A), and non-hypoxia (sample B).

	Sample A (n = 130)	Sample B (n = 59)
Uncharacterized protein (n = 7)	A0A2P9AHZ2 A0A2P9AHG8 A0A2P9ADN6 Q14473	A0A2P9AEL2 Q86T68 Q8ND61
Identical protein (n = 17)	Seven Transmembrane Helix Receptor Molybdopterin Oxidoreductase ABO-Antigen Antigen MLAA-10 Arfaptin-2 Serum Albumin Rheumatoid factor RF-IP24 DNA Helicase Vitamin D-binding protein Epididymis Secretory Sperm Binding protein Alpha-fetoprotein SH3 Domain and Tetratricopeptide Repeat-containing Protein-1 Alpha-2-HS-Glycoprotein Lactoferrin Dynein heavy chain 11, axonemal Nuclear receptor binding SET domain protein 1 A-kinase Anchor Protein 13	
Identified protein	Large neutral amino acids transporter small Probable phospholipid-transporting ATPase VA E3 ubiquitin-protein ligase rififylin Cupin 2 conserved barrel domain protein Synaptotagmin-4 Lumican Interleukin-7	Alternative protein PC Diguanylate cyclase Methylase Protein FAM149A Globin C1 Short-chain dehydrogenase/reductase 3 Sulfotransferase 1A2
	Sample A (n = 130)	**Sample B** (n = 59)
Identified protein	Porimin Transmembrane protein 253 Leucine-rich and calponin homology- domain Inositol-tetrakisphosphate 1-kinase Ankyrin-3 Isochorismatase hydrolase Protein phosphatase 1 Allograft inflammatory factor 1-like Pigment epithelium-derived factor Decorin 4-formylbenzenesulfonate dehydrogenase LBH domain-containing protein 1 C6orf15 PWP2 periodic tryptophan protein homolog TBC1 domain family member 3I Proteosome subunit beta type-11 TCEA2 protein Restriction endonuclease Chromosome 11 open reading frame 65 Hemolysin-type calcium-binding region RAD23 homolog A Olfactory receptor 8J3 CTAGE5 protein Calumenin HRV Fab N27-VL Fibronectin 1 KIR2DL4 Olfactory receptor	Putative ATP-binding cassette sub-family A Actin, cytoplasmic 1 Tripartite motif-containing protein 29 ErfK/YbiS/YcfS/YnhG family protein Ret finger protein-like 3 CD226 antigen Protein Wnt-10b Vasopressin V1a receptor Ornithine cyclodeaminase Zinc finger protein 586 Oligopeptide transporter subunit periplasmic Interstitial collagenase Specifically, androgen-regulated gene protein Low-density lipoprotein receptor-protein 3 Ring finger protein 10 (RNF10), mRNA ABC transporter substrate-binding protein 60S ribosomal protein L19 Coiled-coil domain-containing protein 61 Krueppel-like factor 10 GDNF-inducible zinc finger protein 1 Serine/threonine-protein phosphatase 4 Ubiquitin-like modifier-activating enzyme 1 MDS1 and EVI1 complex locus protein U7 Paired amphipathic helix protein Sin3a Rho GTPase-activating protein 31 Pro-neuregulin-2, membrane-bound isoform Junctophilin 4
	Sample A (n = 130)	**Sample B** (n = 59)
Identified protein	Allantoate amidohydrolase Aminomethyltransferase member-4 mRNA Non-heme chloroperoxidase Vimentin Heterogeneous nuclear ribonucleoprotein D-like Lipopolysaccharide-binding protein Mitogen-activated protein kinase kinase 5 Beta-2-microglobulin Poly(A)-specific ribonuclease PNLDC1 PRPF4 protein variant	Homeobox protein cut-like 1 Thrombospondin 1 Myosin light chain kinase, smooth muscle

Table 1 (*continued*)

	Sample A (n = 130)	Sample B (n = 59)
	Centrosomal protein of 41 kDa	
	cDNA FLJ77412	
	Matrix metallopeptidase 2	
	Metalloproteinase inhibitor 2	
	Insulin-like growth factor-binding protein 4	
	GTP-binding protein 1	
	Zinc finger protein 676	
	Glutathione S-transferase C-terminal domain	
	Carbohydrate sulfotransferase 5	
	ArfGAP with GTPase domain	
	UDP-glucuronosyltransferase 2A1	
	Zinc finger protein 792	
	Asparaginase	
	Poly [ADP-ribose] polymerase 12	
	Sample A (n = 130)	**Sample B** (n = 59)
Identified protein	Oxysterol-binding protein-related protein 11	
	Transcription factor 4	
	Aconitate hydratase, mitochondrial	
	Protein Wnt-11	
	Coiled-coil domain-containing protein 61	
	Retinol-binding protein 4	
	Spermidine/putrescine import ATP-binding	
	Tubulin polyglutamylase TTLL7	
	GEM-interacting protein	
	Zinc finger RNA-binding protein 2	
	Unconventional myosin-XIX	
	Collagen alpha-1(VI) chain	
	Cancer susceptibility candidate 3	
	Collagen type I alpha 2 isoform 1	
	Metalloendopeptidase	
	Transforming growth factor-beta	
	Growth arrest-specific 2 like 3 (Caspase)	
	Fibulin-1	
	Rho GTPase-activating protein 31	
	Cadherin 5 type 2 (VE-Cadherin)	
	Kinesin-like protein KIF27	
	Nuclear receptor coactivator 1	
	Tyrosine-protein kinase receptor Tie-1	
	Coiled-coil domain-containing protein 191	
	G-protein signaling modulator 2	
	Testicular tissue protein Li 61	
	Sample A (n = 130)	**Sample B** (n = 59)
Identified protein	Microtubule-actin cross-linking factor 1	
	Trinucleotide repeat-containing gene 6B protein	
	Extracellular calcium-sensing receptor	
	Transmembrane protein 131	
	Elongator complex protein 2	
	Transport and Golgi organized protein 1 homolog	
	Mediator RNA polymerase-II transcription unit-13	
	Inter-alpha (Globulin) inhibitor H2	
	Cullin-9	
	ATP-binding cassette sub-family A member 9	
	Actinin alpha 1	
	Zinc finger ZZ-type and EF-hand domain-protein 1	
	Host cell factor 1	
	POTE ankyrin domain family member F	
	Laminin subunit alpha-4	
	Leucine-rich repeat and coiled-coil domain	
	Histone-lysine N-methyltransferase 2D	
	Histone-lysine N-methyltransferase 2A	
	Hemicentin-2	
	Mucin-19	
	Protein piccolo	

et al., 2017) The highest protein level was found in hBSC sample A, defined as breastmilk cells secretome-conditioned media under hypoxia. Hypoxic conditions elevate the expansion and differentiation potential of cells due to the up-regulation of telomerase activity in cells and increase the growth and anti-inflammatory molecules of cells (Sancricca, 2010) (Vizoso et al., 2017).

4.4. Proteins identification in the secretome of mesenchymal stem-like cells using Liquid Chromatography-Mass Spectrometry

This experiment discovered a total of 130 proteins (score Sequest HT > 0) from hBSC under hypoxia condition, and 7 of them were uncharacterized. Liquid chromatography-mass spectrometry is a crucial method in proteomic analysis. Proteomic analysis is a methodology that explores the protein composition of distinct macromolecular structures for the individual components, followed by high-resolution mass spectrometry. Sample preparation is the critical step in proteomic analysis. However, the combination of different conditions in the sample lysis process and protein digestion is often the source of sample preparation errors. The chromatogram peaks illustrated various active proteins of samples A and B. Peaks of chromatogram represent components that can be selected based on height or area they cover. The protein conversion to peptides needs efficient depletion of Sodium Dodecyl Sulfate (SDS) from samples. The variety of the sample also impacts digestion efficiency. The exact value of total protein in the sample relates to the amount of complete protein, sample concentration, and protein digestion efficiency (Wiśniewski, 2017).

Previously, proteomic analysis of human milk-derived extracellular vesicles using Western Blotting was conducted. The study identified a total of 1963 protein from 7 donors and found two newly identified milk proteins that were MPZL1 (Myelin protein zero-like protein 1) and EHD3 (EH domain-containing protein 3). They stated that unidentified proteins might relate to the isolation process. Proper isolation is needed for better breastmilk compound separation (Van Herwijnen et al., 2016). Zhang et al. (2016) carried out LC-MS/MS analysis in breastmilk, collected the samples from 4 women at seven-time points over the first six months, and found a total of 247 proteins which 21 of them were significantly changed over lactation. A large number of identified proteins were biologically categorized as LALBA (α-Lactalbumin) and Bile Salt-activated Lipase.

Following the theory by Ballard et al. (2013) and (Verd et al., 2018), Lactoferrin (A0A161I202) and complementary Deoxyribonucleic Acid (cDNA) FLJ54371 that highly similar to Serum Albumin (B4DPP6) were identified in MSC-like secretome. Lactoferrin is an iron-binding glycoprotein and can be found either without bound iron (apo-LF) or in an iron-bound (holo-LF) (Norrby, 2004). Previous studies stated that human and bovine LF could modulate VEGF-A-mediated angiogenesis in vivo (Norrby, 2004; Norrby et al., 2001; Shimamura et al., 2004). Human apo-LF could stimulate angiogenesis in hypoxic states, and the number of VEGF-A receptors would elevate on vascular endothelium (Norrby, 2004). Kim, et al. (2006) found that human LF stimulated VEGF-induced cell proliferation by upregulating KDR/Flk-1 via MAPK activation. apo-LF could perform as normoxic mimetic of hypoxia, capable of maintaining HIF-1α, and would trigger the synthesis of TGF-β (Liao et al., 2012; Zakharova et al., 2012).

This study has two main limitations. The first limitation is that mesenchymal stem cells were not differentiated into osteocyte, chondrocytes, and adipocyte cells. The second limitation is the number of peptide identification obtained in this study was less than in previous studies. The condition is unpredictable because the limit of laboratory capacity and the facility were not appropriate for further identification. However, this study's finding offers a potential effect for novel therapeutic outcomes.

5. Conclusion

The human breastmilk cells contain several mesenchymal stem-like cells and high CD44, CD73, CD90, and CD105 markers on third passage culture. The hypoxic human breastmilk cells secretome produces higher protein levels compared to non-hypoxia. The TGF-β was found in the hypoxic hBSC secretome as a modulator of VEGF-mediated angiogenesis that has a potential effect for novel therapeutics in further studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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