Characterizing the SLC39A14 zinc transporter and collagen products of the chondrocytes from human umbilical cord-derived mesenchymal stromal cell

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ABSTRACT

Background: The SLC39A14 zinc transporter showed a role in the growth of the mouse model. There was no report regarding SLC39A14 protein expression in human chondrocyte, which might prevent further identification of SLC39A14’s function in human growth. This study aims to describe the SLC39A14 protein expression in chondrocyte using human umbilical cord (UC)-derived mesenchymal stromal cell (MSC) isolation and differentiation.

Methods: A cross sectional study was conducted at the Faculty of Medicine, Universitas Padjajaran Bandung, Indonesia, in 2019. The UC was derived after the respondents signed informed consents were documented. Specimens transferred, UC tissue digestion, cell isolation, cultures, and passages were done under the laboratory standard protocols. The immune-phenotyping and differentiation toward chondrocyte procedure were done after third passage. The SLC39A14 expression of the chondrocytes was observed under immunofluorescence microscopy after staining using polyclonal anti SLC39A14 antibody. The collagen 2A and 10A production was measured using the medium supernatant and analyzed using GraphPad Prism 8 edition.

Results: The adherent fibroblast-like cells appeared on day 5th, which continued to proliferate and showed MSC characteristic with positive markers for CD 73, CD90 and CD105, but absent for negative lineage markers. Upon differentiation, cells positive for Alcian blue staining denoted the chondrocyte which shown capacity for further proliferation on Methyl Tetra Toluene assay. Zinc transporter SLC39A14 was expressed on cell membrane. The Collagen 2A produced at a mean level 0.097 ng/ml (SE 0.011;95%CI 0.073-0.12) and Collagen 10A at mean level 31.078 ng/ml (SE 0.923;95%CI 22.870-39.287).

Conclusion: Zinc transporter SLC39A14 was expressed in monolayer human chondrocyte from UC-derived MSC. The chondrocyte shown capacity for proliferation and collagen-producing enables future utility to identify the role of SLC39A14 in chondrocyte pathology.

Keywords: chondrocytes, SLC39A14, collagen 2A, collagen 10A, umbilical cord, mesenchymal stromal cell

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INTRODUCTION

The umbilical cord (UC) has been known as a source for mesenchymal stromal cell (MSC), which can be directed into a differentiation toward chondrocyte, osteocyte, and adipocyte cells.1 Friedenstein was the first who described the supportive hematopoietic cells of bone marrow as MSCs. He exhibited that MSCs could differentiate to bone in vitro and a subset of the cells had a high proliferative potential (CFU-F) when plated at low density in tissue culture.2,3 Based on in vitro study, MSCs is the spindle-shaped plastic-adherent cells isolated from bone marrow, adipose, and other tissue sources, with multipotent differentiation capacity.2,3 Thus, the International Society for Cellular Therapy (ISCT) has promoted that these spindle-shaped, plastic-adherent cells be termed, “mesenchymal stromal cells” of MSCs.4 Many standard procedures have been reported about this process to enable replication.5,6 Based on the multipotent properties of MSCs, it has been known that MSCs can differentiate into chondrocyte cells through a particular growth factor.7-8 A recent report in animal study found that disturbance in zinc (Zn) influx in the growth plate-chondrocytes explained the mechanism of growth failure. This study contributed to a new paradigm primarily related to the SLC39A14 zinc transporter while also highlighted Zn as a ubiquitous metal in human body.9-11 Zn is an important trace element that is involved in various cellular events by regulating the structural and catalytic functions of transcription factors or enzymes.12 Zn homeostasis

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is tightly controlled by two types of Zn transporter such as SLC39/ZIP importers and SLC30/ZnT exporters which is well known participate in various physiological events. Abnormal Zn homeostasis is related to vertebrate growth retardation as well as lead to dwarfism with reductions in the growth-plate width, which is correlated with a decreased of cellular Zn content. The molecules responsible for Zn homeostasis in growth failure have been elusive, and data regarding how Zn affects the intracellular signaling that regulates growth-related endocrine processes still limited. However, a previous study has mentioned that SLC39A14 molecule plays a pivotal role in chondrocyte differentiation through Zn metabolism.

SLC39A14 transporter is knowns as a SLC39/ZIP family member, transports the Zn ion into cells in an in-vitro culture system. A study conducted by Hojyo notified that the PTHr-PKA-CREB signaling pathway of the chondrocyte proliferation phase would not be able to occur when zinc was deficient, leading to growth failure. Using an in-situ hybridization staining procedure, Hojyo and the coworkers identified SLC39A14 molecule in the mouse growth plate-chondrocyte. To date, there was no report regarding SLC39A14 zinc transporter identification in human chondrocyte.

We sought to extend the finding, by identification of the zinc transporter SLC39A14 expression in the human chondrocyte from UC derived MSC. We expect to expand the utilization of the MSC for the chondrocyte differentiation intended for future research in human disease mechanism. Based on those mentioned above, this study aims to characterize the SLC39A14 zinc transporter and collagen products of the chondrocytes from human umbilical cord-derived mesenchymal stromal cell.

**MATERIAL AND METHODS**

**Preparation of material and methods**

The UC derived from Caesarean-delivery, term and non-complicated pregnancy, after exclusion for metabolic and infectious diseases, while a baby born vigorously, with no significant congenital anomaly. The UC derived only after a signed informed consent was documented. All laboratory works were delivered in a sterilized hood started with artery and venous vessels removal to collect the Wharton’s jelly and the remaining tissue. The Collagenase type 1 (Gibco- Nordmark-USA) 3mg/ml was used for enzymatic digestion then incubated for one hour in 5% CO2, 37°C to get the supernatant for the Monolayer cultures with 10 mL DMEM abundant glucose (DMEM enriched with 4.5g/l glucose and L glutamine, 5% human plasma lysate, 2 mM glutamine, also penicillin 100u/ml and streptomycin 100 μg/ml) and MesenPRO RS™ medium.

**MSC culture and harvesting**

Cultures were done in a humidified incubator (Thermo Scientific Forma Series 3 Water Jacketed-CO2 incubator-USA) with 5% CO2 and 37°C temperature environment. The medium was changed in the first 24 hours to remove debris, then every three days afterward, to observe the appearance of separated cells. At the 70-80% confluence, cell was passaged and subcultured until the third passage (P3).

Cells were harvested after P3, followed by cell counting using Trypan blue (Sigma Aldrich-USA). The live-cell was identified from the transparent and shined appearance, while the dead cell would have a broken membrane, so it absorbed the stain, resulting in blue color under a microscope. A number of live cells were calculated according to the formula: live cells in 4 areas/ 4 x 10 dilution x 10⁴ (a constant determined by the manufacturer). Cell viability was calculated using following formula: live cells/total cell x 100%.

**Immunophenotyping**

Immunophenotyping procedure was done with human MSC analysis kit (BD Biosciences-San Jose, CA, USA). The cell suspension was stained with a cocktail of positive marker containing PerCP conjugated anti-human CD105 PerCP-CyTM5.5, APC conjugated anti-human CD73, and FITC conjugated anti-human CD105 PerCP-CyTM5.5, a cocktail of positive marker containing PerCP-erythrin conjugated anti-human CD45/CD34/CD11b/ CD19/ HLA-DR, an isotype match control IgG1κ (MOPC‐21), IgG2ax (G155-178), IgG2bx (27−35), IgM, and IgG3 (A112-3). The analyses were done on a BD FACS-CantoTM II flow cytometer. The BD FACS DivaTM software was used for data reading by gating at 2% isotype control. This procedure was followed by cell count using 0.4% Trypan blue dyes.

**MSC differentiation into chondrocyte**

For differentiation, the MSC was washed then trypsinized using TrypLE™ Express. After incubation, the cells were rewarshed with PBS and resuspended with 0.5 mL Stempro® Chondrogenesis Differentiation Kit™ (Thermo Fisher-life Technologies-USA), in a six-well plate to observe growth and 96 wells plate to optimize cell number in the well for subsequent research procedure. We applied 3200cell/100µL and changed the medium every seven days to avoid damaging the cells, then replace into a basal medium as rapid proliferation was noted. After 21 days, cultures were
fixed and stained with 1% Alcian-Blue to identify chondrocytes under a phase-contrast inverted microscope.

**Methyl Tetra Toluene assay and Immunofluorescence Staining**

We used a 14 days age chondrocyte to carry out Methyl Tetra Toluene (MTT) assay per manufacturer protocol (Thermo Fisher Scientific -USA), Incubation was done at 37°C for 4 hours in the dark, followed by the stop solution (SDS), and read the absorbance at 570 nm. After MTT assay was carried out, we performed the SLC39A14 immunofluorescence staining (Abcam-USA) of the chondrocytes by incubating with the primary antibodies overnight at 4°C, then washed and incubated with diluted secondary antibodies in the dark for one hour. The dilutions of the secondary antibodies (in PBS with 1 wt.% BSA and 0.1 wt.% solution of Tween 20). SLC39A14: Rabbit Anti-Mouse IgG H & L (Alexa Fluor 488) preabsorbed (Abcam-UK) was 1: 1,000. Finally, three drops of the Mounting Medium Fluoroshield with DAPI were added, and the solution was left on the cells for five minutes. Micrographs were taken with an immunofluorescence microscope 40x magnification.

**Collagen 2A-10A measurement and data analysis**

Both Collagen 2A and 10A measurement procedure were based on Sandwich ELISA, using culture medium supernatant according to manufacturer protocol. (LSBio-Seattle, USA). Optical density (OD) value for each well was determined using a microplate reader at 450 nm. Cell morphology was observed with 100x magnificient under an inverted microscope (Observer Z1-Zeiss-Germany), and the image was taken using Axio Visio 4.8 software. A 10 x magnification picture was taken using a hand-phone camera (iPhone 7) for immunofluorescence image. For publication preparation, All images from immunofluorescence procedures were equally edited on Photoshop 8.0 software by mounting 3 layers without any further color changes and no additional editing was done. Data was analyzed and presented on GraphPad Prism 8 edition.

**RESULTS**

Our study found that the stellate cells appeared on 5th day and continue to confluence, when subculture was done, acquiring as much as 1.2175 to 6.65x10⁶ and viability 98.88 to 99.18% MSC, which was confirmed in Figure 1. The immunophenotyping evaluation also showed a positive result for CD73, CD90 and CD105, negative results for hematopoietic lineage and HLA-DR confirming for mesenchymal stromal cells based on ISCT research standards.

Incubation of the MSC in the chondrocyte differentiation medium produced cells with positive staining in 1% Alcian blue (Figure 2). The positive staining confirms the proteoglycan which arises from chondrocyte cells (Figure 2). The initial cell number of 3200 cells per 100 mL yielded a high proliferation on day 10. Hence, we quit using the differentiation medium and used a maintenance medium instead.

A culture on a-24 wells-plate was done to identify SLC39A14 membrane expression shown positive staining on immunofluorescence.
The human umbilical cord has raised more attention in recent years as a source for the MSC. The ISCT defined minimum criteria for the MSC based on the ability to adhere to plastic dish, positivity on CD73, CD90 and CD105 and negative for CD34, CD45, CD11b, CD19, and HLA-DR, as well as ability for differentiation. This study samples showed 95.7% to 97% positivity for the MSC surface antigen and 3.5% for negative lineage, and ability to differentiate into the chondrocyte. This result determined the isolation of umbilical tissue stromal cells were the MSC.

The chondrocyte which resides typically within a lacuna and embedded in the proteoglycans matrix in the growth plate also shown similar inhabitation while in the monolayer culture in our study. The confirmatory 1% Alcian blue staining even shown the proteoglycans enhancement, including matrix fibril accentuated from the chondrocytes. Our study also shown similar morphology to the study by Oppenheimer et al.

The zinc transporter 14 or SLC39A14 protein was a member of the solute carrier family. This carrier was used together with iron, hence getting the name zinc/iron transporter (ZIP), although having more functional role in different cells, such as for manganese influx. The disturbance of SLC39A14 prevented zinc influx in the chondrocyte, hence, interfered different pathways resulting in acceleration of hypertrophic phase as documented in various studies.

Our 12 independent samples showed mean Collagen 2A concentration was at 0.097 ng/ml (SE 0.011; 95%CI 0.073-0.12) and mean Collagen 10A level was 31.078 ng/ml (SE 3.792;95%CI 22.870-39.287), as shown in Figure 4. These results indicate that based on the average value, the Collagen 10A level was higher compared with Collagen 2A concentration after 7 days on the differentiation medium and 3 days on maintenance medium (Figure 4).

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The zinc transporter SLC39A14 was recognized in the hypophysis, liver and growth plate chondrocyte by Hojyo and co-worker in mouse model using in-situ hybridization. We used primary culture cell from human UC and provided evidence to identify the SLC39A14 in human chondrocyte using an immunocytochemistry staining. We could not able to find any literature mentioned identification of the SLC39A14 in human chondrocyte previously, hence this study presumably the first. To visualize the immunofluorescence antigen-antibody complex better, we used fluorophore-conjugated antibodies (Alexa Fluor). The staining procedure allowed us to identify membranous location of the SLC39A14 protein on the chondrocyte while the nucleus was identified separately on a counterstaining method with DAPI. This localization would support future research regarding zinc role in the chondrocyte as a continuum for the similar studies in animal.

While on monolayer culture, our study showed the chondrocyte produced the collagen, such as collagen type 2A (col2a1) which is typical for the proliferation zone or collagen type 10A (col10A1) in the hypertrophic zone. These products could...
be used as markers to identify which phase of the differentiation was occurring. These collagen 2A1 and collagen 10A1 were in accordance with Hojyo result also to the study by Yuan et al., using human-cartilage-chondrocyte.9,15 A study conducted by Yuan et al. found that the human UC as a source gave advantage from low risk of environmental exposure or disease compared to adult cartilage.32

CONCLUSION

This study showed the identification of SLC39A14 zinc transporter in chondrocyte from human UC derived-MSC differentiation and the collagen product. This attempt provided an alternative option for a self-engineered-chondrocyte in cell research.

ETHICAL CONSIDERATION

This study was a part of research: Role of HIV-1 Tat protein to the chondrocyte SLC39A14-zinc transporter and zinc utility as the mechanism of stunting in children with HIV infection. Informed consent for participating in umbilical cord contribution for the research was derived from parents of umbilical cord donors. Ethical Committee approved this experiment at the Faculty of Medicine, Universitas Padjadajaran number: 205/UN6.KEP/EC/2018, dated March 20th 2018.

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

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AUTHORS CONTRIBUTION

Ketut Dewi Kumara Wati, Meita Dhamayanti, Budi Setiabudiawan, Ida Parwati develop the research plan, define intellectual content, proposed grant budget, data interpretation, build the manuscript, Endah Dianty Pratiwi, Yanni Dirgantara, Cynthia Retna Sartika develop laboratory methods and management, Endah Dianty Pratiwi, Yanni Dirgantara, Ketut Dewi Kumara Wati, execute laboratory work. All authors discussed the results and contributed to the final manuscript.

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REFERENCES

1. Matthy MA, Pati S, Lee JW. Concise Review: Mesenchymal Stem (Stromal) Cells: Biology and Preclinical Evidence for Therapeutic Potential for Organ Dysfunction Following Trauma or Sepsis. Stem Cells. 2017;35(2):316-324.
2. Friedenstein AJ, Petroukova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968; 6(2):230-47.
3. Friedenstein AJ, Deriglosava UF, Kulagina NN, Panasuk AF, Rudakova SE, Luriá EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol. 1974; 2(2):83-92.
4. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy. 2005;7(5):393-5.
5. Ullah I, Subbarao RB, Rho GJ. Human mesenchymal stem cells - current trends and future prospective. Biosci Rep. 2015;35(2).
6. Bocic, Richter W. Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. Stem Cell Res Ther. 2010;1(4):31.
7. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res. 1998;238(1):265-72.
8. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001;268(2):189-200.
9. Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, Koseki H, et al. The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. PLoS One. 2011;6(3):e18059.
10. Maret W. Zinc biochemistry: from a single zinc enzyme to a key element of life. Adv Nutr. 2013;4(1):82-91.
11. Seklier I, Sensi SL, Hershinkel M, Silverman WF. Mechanism and regulation of cellular zinc transport. Mol Med. 2007;13(7-8):337-43.
12. Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. J Biol Chem. 2006;281(34):34085-9.
13. King JC, Woodhouse, LR. Zinc homeostasis in humans. J Nutr. 2000; 130(5S Suppl):1360S-6S
14. Prasad AS. Zinc: an overview. Nutrition. 1995;11(1 Suppl):93-9.
15. Lichten LA, Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr. 2009;29:153–176.
16. Rossii L, Migliaccio S, Corsi A, Marzia M, Bianco P, et al. Reduced growth and skeletal changes in zinc-deficient growing rats are due to impaired growth plate activity and inanition. J Nutr. 2001;131(4):1142–1146.
17. Taylor KM, Morgan HE, Johnson A, Nicholson RI. Structure-function analysis of a novel member of the LIV-1 subfamily of zinc transporters, ZIP14. FEBs Lett. 2005;579(2):427–432.
18. ThermoFisher Scientific. Gibco MesenPRO RSMSTM Medium. 2010. [Available from https://assets.thermofisher.com/TFSAssets/LSG/manuals/MesenProRSM_man.pdf]. [Accessed March 27, 2019]
19. BD Stemflow hMSC Analysis Kit. BD bioscience. 2009. [Available from: https://www.bdbiosciences.com/documents/BD_Stemflow_hMSC_Analysis_Kit.pdf] [Accessed on March 13th 2019]

20. StemPRO™ Chondrogenesis Differentiation Kit. Available from: https://www.thermofisher.com/order/catalog/product/A1007101. Accessed on March 27th 2019.

21. Vybrant MTT Cell Proliferation Assay Kit. 2010. [Available from: https://www.thermofisher.com/id/en/home/references/protocols/cell-culture/mtt-assay-protocol/vybrant-mtt-cell-proliferation-assay-kit.html] [Accessed on March 27th 2019]

22. Abcam Immunocytochemistry-immunofluorescence protocol. Abcam. 2012. [Available from: https://www.abcam.com/protocols/immunocytochemistry-immunofluorescence-protocol] [Accessed on March 13th 2019]

23. Abcam secondary antibodies alexa fluor 488 conjugated antibodies. 2013. [Available from: https://www.abcam.com/secondary-antibodies/alexa-fluor-488-conjugated-antibodies] [Accessed on March 13th 2019]

24. Human collagen 10A1/Collagen X ELISA KIT (Sandwich Elisa) User Manual. 2013. [Available from: http://www.lsbio.com] [Accessed on March 27th 2019]

25. Human CTX II ELISA KIT (Sandwich Elisa) User Manual. 2012. [Available from: http://www.lsbio.com] [Accessed on March 27th 2019]

26. Kozhemyakina E, Lassar AB, Zelzer E. A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. Development. 2015;142(5):817-31.

27. Maity B, Sheff D, Fisher RA. Immunostaining: detection of signaling protein location in tissues, cells and subcellular compartments. Methods Cell Biol. 2013;113:81-105.

28. Tuschl K, Meyer E, Valdivia LE, Zhao N, Dadswell C, Abdul-Sada A, et al. Mutations in SLC39A14 disrupt manganese homeostasis and cause childhood-onset parkinsonism-dystonia. Nat Commun. 2016;7:11601.

29. Oppenheimer H, Meir H, Haze A, Kandel L, Leibergall M, Dvir-Ginzberg M. THU0033 SET7/9 and SIRT1 induce promoter-driven collagen 2ALPHA1 expression in 3D cultured human chondrocytes. Ann Rheum Dis. 2014;71(3):348-60.

30. Rodríguez JP. Effects of zinc on cell proliferation and proteoglycan characteristics of epiphyseal chondrocytes. J Cell Biochem. 2001;82(3):501-11.

31. Wang XFG, Gay CV, Leach-Jr RM. Short-term zinc deficiency inhibits chondrocyte proliferation and induces cell apoptosis in the epiphyseal growth plate of young chickens. J Nutr. 2002;132(4):665-73.

32. Yuan Y, Tan H, Dai P. Krüppel-like factor 2 regulates degradation of type II collagen by suppressing the expression of matrix metalloproteinase (MMP)-13. Cellular Physiology and Biochemistry. 2017;42(6):2159-68.

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