Recombinant laminin α5 LG1-3 domains support the stemness of human mesenchymal stem cells

SUJIN LEE[1*], DONG-SUNG LEE[2*] and JUN-HYEOG JANG[1]

[1]Department of Biochemistry, College of Medicine, Inha University, Incheon 22212;
[2]College of Pharmacy, Chosun University, Gwangju 61452, Republic of Korea

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Abstract. The extracellular matrix components laminin and elastin serve key roles in stem cell therapy. Elastin-like polypeptides (ELPs), derived from a soluble form of elastin, affect the proliferation and differentiation of various types of cells. In the present study, a novel protein was designed containing globular domains 1-3 of laminin α5 (Lα5LG1-3) fused to ELPs (Lα5LG1-3/ELP). Lα5LG1-3/ELP was expressed in *Escherichia coli* and displayed a molecular size of ~70 kDa on 12% SDS-polyacrylamide gels. The cellular activities, such as cellular adhesion (adhesion assay) and proliferation (MTT cytotoxicity assay), of human mesenchymal stem cells (hMSCs) treated with 1 μg/ml of Lα5LG1-3/ELP were enhanced compared with those of untreated cells. Additionally, the number of undifferentiated hMSCs and their degree of stemness were assessed based on the gene expression levels of the stem cell markers cluster differentiation 90 (CD90), endoglin (CD105) and CD73. The expression levels of these markers were upregulated by 2.42-, 2.29- and 1.92-fold, respectively, in the hMSCs treated with Lα5LG1-3/ELP compared with the levels in untreated controls. Thus, Lα5LG1-3/ELP may be used to enhance the viability of hMSCs and preserve their undifferentiated state, whereby the clinical applications of hMSCs may be improved.

Introduction

One of the key treatments in regenerative medicine is stem cell therapy. Thus, the maintenance of stem cells is essential. Maintenance of the undifferentiated state and proliferation activity of stem cells is essential to sustain their functionality (1). It has been hypothesized that this requirement can be met by mimicking the *in vivo* extracellular matrix (ECM) configuration, thereby modulating the activity of stem cells *in vitro* (2). The principle behind this hypothesis is that the ECM not only functions as structural support for stem cells *in vivo* but also provides biochemical cues for their maintenance versus directed differentiation (3).

Basement membranes (BMs) are a subgroup of the ECM that is necessary for cell differentiation during early developmental processes. In addition, BMs are critical for the formation and maintenance of mature tissues (4,5). Laminin, one of the components of BMs, consists of three genetically distinct subunits called α, β and γ chains, which are assembled into cross-shaped molecules (6,7). At present, 5α, 3β and 3γ chains, as well as at least 16 different trimeric laminin isoforms are known in humans and mice each (8). Laminin-mediated cell attachment and cellular behavior are achieved by five different α chains. Among them, laminin α5 (Lα5) plays a key role during embryogenesis. Lα5, expressed in the inner cell mass of the blastocyst, supports the self-renewal of embryonic stem cells (7,9). Lα5-knockout mouse or zebrafish models have severe developmental defects, demonstrating the effect of Lα5 on stem cell maintenance and embryonic development (10). Most of the cellular binding regions of laminins are primarily located in the C-terminal large globular (G) domain of the protein. The G domain consists of only the laminin α chain and is subdivided into five homologous LG domains (4,11). LG domains are important for interaction with cells (12). LG1, LG2 and LG3 are the first three of the LG domains, assembled into a clover-leaf arrangement to form the putative binding sites of laminin-binding integrin (11). LG1-3 domains of the α5 chain comprise cell-binding sites, which is mediated by integrins α3β1, α6β1 and α6β4. LG4 domain of the α5 chain binds to α-dystroglycan and heparin (12-14).

Elastin is an important ECM protein found abundantly in blood vessels and serves as a biomechanical and physiological signal for cells (15). The soluble form of elastin, tropoelastin, is primarily located in elastic tissues. It has been reported to promote the proliferation of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (16). Additionally, it is often used in the form of cross-linked gel fibers or injectable scaffolds for tissue engineering and drug delivery (17). Elastin-like polypeptides (ELPs) are recombinant peptide polymers composed of VPGXG pentapeptides (found in tropoelastin), where X stands for any amino acid residue except

Correspondence to: Professor Jun-Hyeog Jang, Department of Biochemistry, College of Medicine, Inha University, 100 Inha, Michuhol, Incheon 22212, Republic of Korea
E-mail: juhjang@inha.ac.kr

*Contributed equally

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proline (18). ELPs exhibit biocompatibility that is similar to elastin in terms of mechanical and viscoelastic properties. ELPs effectively modulate the migration, proliferation, and differentiation of various type of mammalian cells (19). These polypeptides can be genetically engineered to modulate their structural and biological properties and exhibit an inverse phase transition behavior in response to the changes in their solutions (20). Recombinant ELPs have been expressed in *E. coli* and subsequently purified through a separation process known as inverse transition cycling (ITC) (21).

Human MSCs (hMSCs) are pluripotent stem cells that can differentiate both *in vitro* and *in vivo*. In particular, it is known that hMSCs can differentiate into the mesenchymal lineage, forming bone, cartilage, adipose, and muscle cells. These stem cells can be isolated from cord blood, placental fluids, and multiple adult tissues, such as bone marrow, adipose tissue, skeletal muscle, and connective tissue (22-27). Previously, we have shown that a recombinant human laminin α2 LG1-3 promotes hMSCs physiology *in vitro*, including cell adhesion, proliferation, and stemness (28). In this study, we fused the coding sequence of ELP to laminin α5 LG1-3 domains and examined the cellular effects of this fusion construct (Lα5LG1-3/ELP) on hMSCs.

Materials and methods

Construction and purification of Lα5LG1-3/ELP fusion protein. Lα5LG1-3 coding sequence containing integrin-binding modules LG1-LG3 of laminin α5 (Lα5) and ELP coding sequence were synthesized (Genotech).

Based on the characteristics of ELP guest residues (19-21), ELP coding sequence was designed by inserting Val, Leu, and Gly (ratio of 17:4:9) into ELP guest residue position. The full-length ELP(V17L4G9-30) coding sequence containing Val, Leu, and Gly in the fourth guest residue (Xaa) (Val-Pro-Gly-Xaa-Gly) of the pentapeptide repeat is VPGGG VPGVG VPGGG VPGVG VPGVG VPGGG VPGVG VPGVG VPGGG VPGLG VPGVG VPGVG VPGGG VPGVG VPGLG VPGVG VPGVG VPGGG VPGVG VPGLG VPGVG VPGVG VPGGG VPGVG VPGLG VPGVG VPGVG VPGGG VPGVG VPGGG.

The Lα5LG1-3 construct was double digested with *Sac*I and *Kpn*I and then cloned into the expression vector pBAD-His (Invitrogen; Thermo Fisher Scientific, Inc.) to construct the pBAD-His-Lα5LG1-3. Next, ELP construct was double digested with *Kpn*I and *Eco*RI and then cloned into pBAD-His-Lα5LG1-3 to construct the pBAD-His-Lα5LG1-3/ELP.

The recombinant pBAD-His-Lα5LG1-3/ELP was transformed into *E. coli* TOP10 cells through heat shock at 42˚C. A single colony was selected and inoculated into 20 ml Luria-Bertani (LB) media (LPS Solution) containing 100 μg/ml ampicillin (LB-Amp) overnight at 37˚C. This overnight culture was then mixed with 1 liter LB medium and incubated until the OD_{600} reached 0.4. Subsequently, L-Arabinose was added to a final concentration of 0.1% (w/v), and the culture temperature was decreased to 20˚C. After 6 h of induction, bacterial cells were pelleted by centrifugation at 4˚C and 6000 x g for 15 min. The pellet was resuspended in sodium chloride-Tris-EDTA buffer and then lysed by sonication. The lysate was obtained by centrifugation at 13,000 x g and 4˚C for 20 min. To obtain Lα5LG1-3/ELP, the supernatant was purified through ITC. Briefly, the supernatant was transferred into fresh tube with 3 M NaCl pre-heated to 40˚C, followed by centrifugation at 40˚C to obtain the Lα5LG1-3/ELP pellet. After discard of supernatant containing soluble Lα5LG1-3/ELP, and the pellet was re-solubilized in cold phosphate-buffered saline. The resulting solution was centrifuged at 4˚C to remove any insoluble protein, and the supernatant, containing pure Lα5LG1-3/ELP, was kept. The purity of this solution was analyzed through 12% SDS-PAGE, followed by Coomassie blue staining. Additionally, western blots analysis was performed using a peroxidase-conjugated monoclonal anti-polyhistidine antibody (His antibody, sc-8036 HRP; Santa Cruz Biotechnology, Inc.) diluted at 1:1,000 to assess the expression of Lα5LG1-3/ELP. The size of the immunodetected protein was determined based on the pre-stained protein markers (Elpis Biotech) electrophoresed in parallel lanes.

Cell culture. Primary hMSCs were kindly provided by Dr Sung-Won Kim (St. Mary's Hospital, the Catholic University of Korea) (29,30). The cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and cultured in the growth medium (minimum essential medium, α-modification) (α-MEM) (Welgene) containing 10% fetal bovine serum (Welgene), 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B (Invitrogen; Thermo Fisher Scientific, Inc.). Confluent cells were passaged by trypsinization (0.25% trypsin-EDTA solution for 3 min). Cells were maintained for three passages before use for further cell experiments.

Adhesion assay. Cell adhesion assay was carried out using crystal violet. Culture plates were coated with 0, 0.01, 0.05, 0.1, 0.5, 1.0, or 5.0 μg/ml of purified Lα5LG1-3/ELP protein for 2 h at 37˚C. Following three rinse with Dulbecco's phosphate-buffered saline (DPBS) (Welgene), the plates were blocked with 0.5% (w/v) bovine serum albumin (BSA) (Gibco; Thermo Fisher Scientific, Inc.) in DPBS for 1 h at 37˚C. Subsequently, hMSCs were added ($5x10^4$ cells/well) and incubated at 37˚C for 30 min. Three replicates were performed for each treatment. After incubation, non-attached cells were removed by three rinses with DPBS, and the remaining cells were fixed by incubation in 3.7% formalin solution for 20 min at room temperature. The fixed cells were stained with 0.25% (w/v) crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Subsequently, they were extensively rinsed with DPBS and then lysed using 2% SDS. The culture plates were read on a microplate reader (BioTek) at 570 nm. Additionally, cell adhesion was monitored with 15 min intervals to confirm the adhesion over time. Cell adhesion activity was normalized relative to uncoated plates.

MTT cytotoxicity assay. Twenty-four-well plates were coated with 1 μg/ml Lα5LG1-3/ELP protein for 2 h at 37˚C and then rinsed three times with DPBS. Subsequently, hMSCs were added ($1x10^4$ cells/well) and incubated for 7 days at 37˚C. Three replicates were performed, and cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; AMRESCO Inc.) assay, which measures the metabolic activity of viable cells, according to the manufacturer's directions (Promega). Briefly, cells were rinsed three times with DPBS and then incubated with 0.5 ml/well of

Table I. Sequences of the primers used for reverse transcription-quantitative PCR.

Gene	Forward primer	Reverse primer
β-actin	5'-TGGCACCCAGCACAATGAAGAT-3'	5'-TACTCCTGCTTGCTGATCCA-3'
CD90	5'-CCAAAGGCTTCTTCTTGCTG-3'	5'-CCACCAAATGTGAAGACGTG-3'
CD105	5'-GAAAATGGAGCTCCTGGTCA-3'	5'-ACCCTTAGCACCAACAGCAC-3'
CD73	5'-CAGTACCAGGGCACTATCTGG-3'	5'-AGTGGCCCCTTTGCTTTAAT-3'

CD, cluster of differentiation.

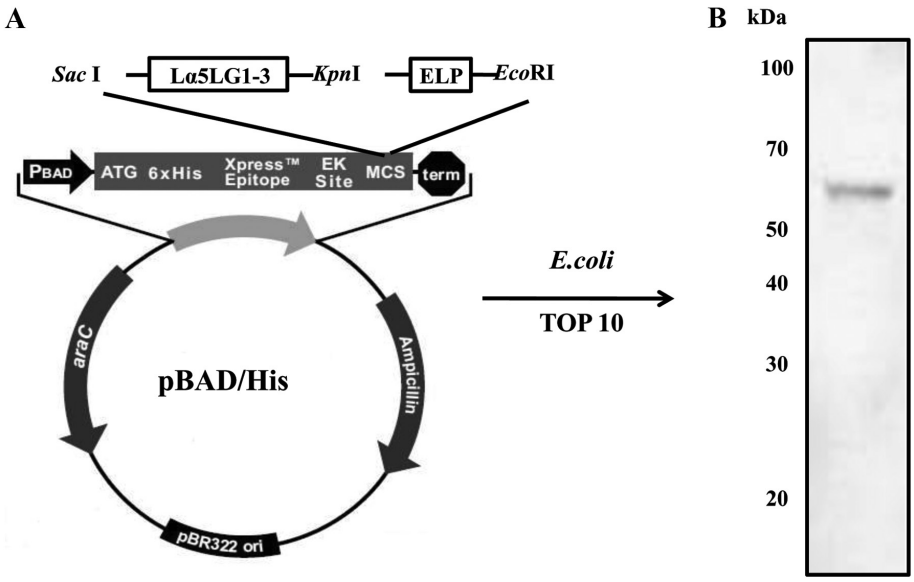

Figure 1. Cloning and purification of recombinant Lα5LG1-3/ELP. (A) cDNA encoding ELP was cloned into the pBAD-HisA-Lα5LG1-3 expression vector. (B) Molecular weight of the protein was estimated as 70 kDa via western blotting probed using an anti-His6-tag monoclonal antibody. Lα5LG1-3/ELP, globular domains 1-3 of laminin α5/elastin-like polypeptide.

5 μg/ml MTT in DPBS for 2 h at 37˚C. Afterward, the MTT solution was removed, and the resulting formazan crystals were dissolved in 150 μl/well of dimethyl sulfoxide. The plates were read at 570 nm on a microplate reader (BioTek). Data were normalized using the uncoated control.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The total RNA of the cell was isolated using the Easy-Spin Total RNA Extraction Kit and then purified following the manufacturer's directions (Intron). The RNA amount and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Next, cDNA was synthesized from 1 μg of total RNA by using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's directions (Applied Biosystems; Thermo Fishier Scientific, Inc.). qRT-PCR was performed using SYBR-Green PCR Master Mix (Toyobo) and the ABI Step One Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR conditions were as follows: 10 min at 95˚C and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The gene expression level was normalized to that of β-actin and estimated with the comparative C_q method (31). Each experiment per sample was performed in triplicate. The primers used are listed in Table I.

Statistical analysis. ANOVA followed by a post-hoc Tukey's test was used for comparisons between various groups. Differences between two groups were tested via unpaired Student's t-test: P-value <0.05 was considered significant (*P<0.05, **P<0.01 and ***P<0.001). Experiments were repeated three times, and the results are presented as mean ± SD.

Results

Expression and purification of Lα5LG1-3/ELP protein. To make the recombinant Lα5LG1-3/ELP construct, the Lα5LG1-3 sequence was inserted into the pPAD-HisA expression vector. Then, the ELP sequence was inserted into the resulting pBAD-His-Lα5LG1-3 expression vector. The Lα5LG1-3/ELP fusion protein retained the phase transition property of ELP. Thus, Lα5LG1-3/ELP could be purified through ITC. The molecular size of Lα5LG1-3/ELP was estimated at approximately 70 kDa on 12% SDS-polyacrylamide gels stained with Coomassie blue. The expression of Lα5LG1-3/ELP was verified by western blotting with a peroxidase-conjugated monoclonal anti-polyhistidine antibody specific to the N-terminal His-tag (Fig. 1). Production of this Lα5LG1-3/ELP protein was well-established and used in subsequent experiments.

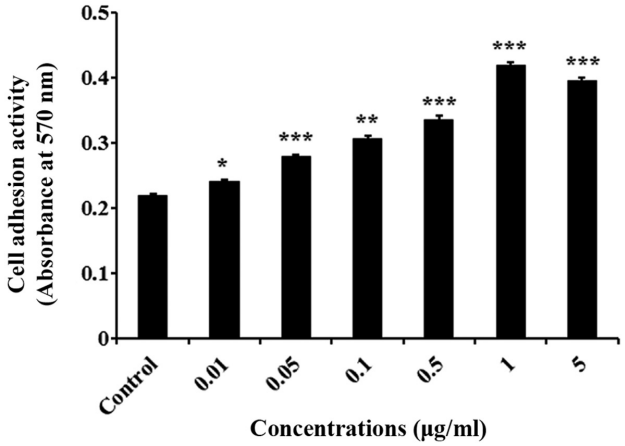

Figure 2. Adhesion of human mesenchymal stem cells in plates coated with recombinant Lα5LG1-3/ELP. Attached cells were observed using the crystal violet assay, and the results are expressed as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control. Lα5LG1-3/ELP, globular domains 1-3 of laminin α5/elastin-like polypeptide.

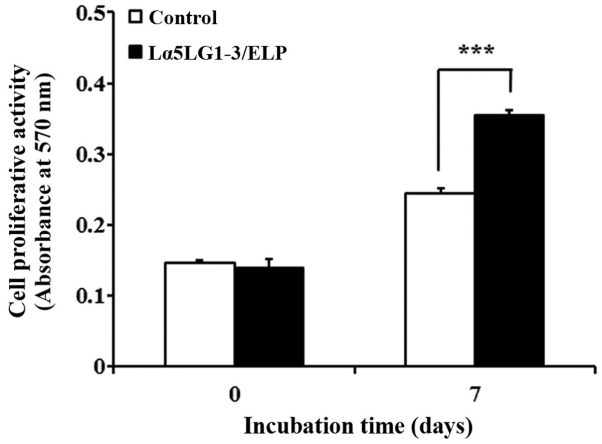

Figure 3. Effect of recombinant Lα5LG1-3/ELP on the proliferation of human mesenchymal stem cells evaluated using MTT assay. The results are expressed as the mean ± SD of three independent experiments. ***P<0.001. Lα5LG1-3/ELP, globular domains 1-3 of laminin α5/elastin-like polypeptide.

Lα5LG1-3/ELP promotes the adhesion of hMSCs. Cell adhesion assay was carried out using crystal violet to determine the effect of Lα5LG1-3/ELP on the adhesion of hMSCs. As shown in Fig. 2 (P<0.001), the attachment of hMSCs was significantly better in the wells coated with Lα5LG1-3/ELP than in the uncoated, control wells. Moreover, Lα5LG1-3/ELP improved the cell adhesion in a dose-dependent manner, with the cell adhesion saturated at 1 μg/ml of Lα5LG1-3/ELP. Therefore, Lα5LG1-3/ELP was used at 1 μg/ml in the subsequent experiments.

Lα5LG1-3/ELP enhances the proliferation of hMSCs. We measured the effect of Lα5LG1-3/ELP on the proliferative activity of hMSCs by using the MTT assay. Toward this end, 24-well plates were coated with Lα5LG1-3/ELP, and hMSCs were incubated in these coated wells for 7 days. Additionally, experiments were conducted in two groups of 0 and 7 days for comparison. After 7 days, the proliferation of hMSCs increased 1.45-fold (P<0.001; Fig. 3) in the coated well compared with the proliferation in the

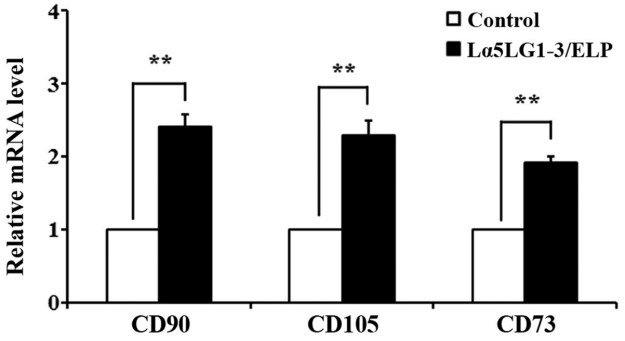

Figure 4. Recombinant Lα5LG1-3/ELP enhances the stemness of human mesenchymal stem cells. The mRNA expression levels of CD90, CD105 and CD73 were measured using reverse transcription-quantitative PCR. The mRNA expression levels of each gene were normalized relative to the mRNA levels of β-actin, which served as an internal control. The results are expressed as the mean ± SD of three independent experiments. **P<0.01. Lα5LG1-3/ELP, globular domains 1-3 of laminin α5/elastin-like polypeptide; CD, cluster of differentiation.

controls (uncoated wells). This result demonstrated that Lα5LG1-3/ELP enhances the proliferation of hMSCs.

Lα5LG1-3/ELP helps maintain the stemness of hMSCs. qRT-PCR was performed to confirm that Lα5LG1-3/ELP maintains stemness of hMSCs at the gene expression level. The distinctive stem cell surface marker genes cluster differentiation 90 (*CD90*), endoglin (*CD105*) and *CD73* were chosen. The mRNA levels of these markers in hMSCs grown with or without Lα5LG1-3/ELP for 7 days are presented in Fig. 4. The gene expression levels of *CD90*, *CD105* and *CD73* were upregulated in the presence of Lα5LG1-3/ELP compared with the levels in the uncoated control wells.

Discussion

Cells must interact with the ECM because it supports many cellular activities, including adhesion, proliferation, and differentiation (32). Laminin and elastin are two ECM proteins predominantly present in the BMs of most tissues in humans (33). The major multifunctional components of the ECM include the followings: i) Laminin is one of the glycoproteins and a major factor affecting cell attachment, proliferation, differentiation and survival. The five globular domains of the α-chain, LG domains, are known to have a critical effect on cells by binding to integrins. ii) Elastin affects the biomechanical and physiological properties of cells (32,33). In this work, we engineered a novel fusion protein composed of LG1-3 domains laminin α5 chain and ELPs, recombinant forms of elastin.

The Lα5LG1-3/ELP fusion protein was purified through ITC, based on the inverse phase transition properties of ELP. As shown in Fig. 1, the total molecular weight of the recombinant protein was estimated at approximately 70 kDa by performing western blot analysis with and antibody that detects the amino-terminal His-tag. The production of the recombinant protein was successful, and the presence of Lα5LG1-3/ELP was determined by protein identification. The major BM proteins laminins, exert biological activities, such as promoting cell attachment, differentiation, and migration, as well as angiogenesis and formation of neurite outgrowths (5,6).

Integrins are the most characteristic laminin receptors that the diverse biological activities of laminins. Moreover, interactions of cells with laminins are crucial to cellular adhesion (4,33).

The hMSCs are pluripotent stem cells that can differentiate into mesenchymal lineages (22,23). To assess the effect of Lα5LG1-3/ELP on the adhesion of hMSCs, we performed the cell adhesion assay by using crystal violet. Lα5LG1-3/ELP enhanced the adhesion of hMSCs in a dose-dependent manner, with saturation at 1 μg/ml. Therefore, Lα5LG1-3/ELP, was used at the concentration of 1 μg/ml for the subsequent experiments (Fig. 2). To further estimate the effect of Lα5LG1-3/ELP on hMSC proliferation, MTT assay was carried out. For this purpose, hMSCs were grown in Lα5LG1-3/ELP coated 24-well plates for 7 days and then used for the assay. In addition, for comparison, experiments were conducted in two groups, 0 day and 7 day. As shown in Fig. 3, after 7 days, the cell proliferation was 1.45-fold higher in the coated plates than in the uncoated controls. Taken together, these results suggest that Lα5LG1-3/ELP improves the proliferation of hMSCs.

It has been reported that hMSCs express CD44, CD71, CD73, CD90 (Thy-1), CD105 (endoglin), and CD271, but not the co-stimulatory molecules CD80, CD86 and CD40, or the hematopoietic markers CD14, CD34 and CD45 (22). In addition, hMSCs express various surface receptors, such as integrin, that bind to the LG1-3 domain of the laminin α-chain. Therefore, hMSCs are anticipated to bind to recombinant laminin α5 LG1-3 (Lα5LG1-3) (27). To investigate the effect of Lα5LG1-3/ELP on the maintenance of the stemness properties of hMSCs, the gene expression levels of the stem cell surface markers CD73, CD90 and CD105 were measured through qRT-PCR. Total RNA was isolated from cells cultured in Lα5LG1-3/ELP coated plates for 7 days, cDNA was synthesized and surface markers were examined, and CD90, CD105, and CD73 were stem cell surface markers that are expressed by hMSCs at varying levels (34). CD90 is a stem cell surface-anchoring glycoprotein that plays a key role in cell motility, and is essential for stem cell growth and differentiation (35,36). CD90 expression at high levels is associated with the undifferentiated state of MSCs (37). CD105, also called SH2, is a component of a receptor complex that transforming growth factor-β (TGF-β), which modulates stem cell migration, proliferation, and differentiation. Additionally, CD105 is also used for purifying hMSCs through an immunoisolation method. CD105-positive MSCs can differentiate into chondrogenic, osteogenic, and adipogenic lineages (36,38,39). CD73, also termed as ecto-5'-nucleotidase, is a glycosyl phosphatidylinositol-linked membrane protein found on the surface of multiple cell types. It is found on the surfaces of hematopoietic and mesenchymal stem cells and functions in numerous physiological processes in various tissues (40-42). In addition, CD73 is known to modulates epithelial-mesenchymal stem cell transition and stemness in ovarian cancer initiating cells (43). As shown in Fig. 4, the expression levels of the surface markers CD90, CD105 and CD73 were 2.42-, 2.29- and 1.92-fold higher, respectively, than the levels in the control. These results indicate that Lα5LG1-3/ELP contributes to maintaining the undifferentiated state of hMSCs. The increase in CD90, CD105 and CD73 levels in hMSCs cultured in Lα5LG1-3/ELP-coated plates suggest that Lα5LG1-3/ELP supports the stemness properties of hMSCs. In order to further clarify the effect of Lα5LG1-3/ELP, and experiment at the protein level such as western blot will be performed later.

In conclusion, Lα5LG1-3/ELP maintains the undifferentiated state of hMSCs and promotes their adhesion, proliferation, and stemness. Therefore, Lα5LG1-3/ELP can potentially be used during MSC therapy to enhance the viability of these stem cells and their maintenance at the undifferentiated state. Hence, *in vivo* experiments with recombinant laminin are required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL, DSL and JHJ conceptualized the study. SL and JHJ analyzed the data, performed the experiments and curated the data. SL, DSL and JHJ prepared and wrote the original manuscript, reviewed and edited the manuscript, including the figures. JHJ supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The human mesenchymal stem cells were isolated from tissues discarded during surgery. The study was conducted with the written informed consent of the subject who provided the tissue. The isolation of human mesenchymal stem cells from tissues was approved by the Institutional Review Board of the Catholic University of Korea, St. Mary's Hospital (approval no. KC08TISS034). All cells were provided by Dr Sung-Won Kim (St. Mary's Hospital, the Catholic University of Korea).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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