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Modification of Pluripotency and Neural Crest-Related Genes' expression in Murine Skin-Derived Precursor Cells by Leukemia Inhibitory Factor (LIF)

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Skin-derived precursor cells (SKPs) are multipotent, sphere-forming and embryonic neural crest-related precursor cells that can be isolated from dermis. It is known that the properties of porcine SKPs can be enhanced by leukemia inhibitory factor (LIF) which is an essential factor for the generation of embryonic stem cells in mice. In our present study, to enhance or maintain the properties of murine SKPs, LIF was added to the culture medium. SKPs were treated with 1,000 IU LIF for 72 hours after passage 3. Quantitative real time RT-PCR was then performed to quantify the expression of the pluripotent stem cell specific genes *Oct4, Nanog, Klf4* and *c-Myc*, and the neural crest specific genes *Snai2* and *Ngfr*. The results show that the expression of *Oct4* is increased in murine SKPs by LIF treatment whereas the level of *Ngfr* is decreased under these conditions. Interestingly, LIF treatment reduced *Nanog* expression which is also important for cell proliferation in adult stem cells and for osteogenic induction in mesenchymal stem cells. These findings implicate LIF in the maintenance of stemness in SKPs through the suppression of lineage differentiation and in part through the control of cell proliferation.

Key words: Skin precursor cell, Leukemia inhibitory factor, Oct4, Nanog, Ngfr

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Introduction

Stem cells have properties of self-renewal and various lineage differentiations in the body [1,2]. Recently, variety of multipotent stem or precursor cells was found in the region of skin such as bulge, hair follicle and dermis [3,4]. Among them, skin-derived precursor cells (SKPs) are originated from juvenile and adult dermis in mammals. In SKPs, *Sox2*, *Klf4* and *c-Myc*, which are key factors for induced pluripotent stem cells [5], and *Snai2* and *Ngfr*, neural crest-related genes, are highly expressed and they have both properties of stem cells and neural crest cells [6-8]. SKPs are capable of differentiating neural and mesodermal lineage cells in human, mouse and pig, corresponding to the multipotency of embryonic neural crest stem cells [4,7-10]. The cells are isolated from the dermis and cultured *in vitro* as suspending condition in the existence of bFGF and EGF. SKPs can be differentiated into neurons, glia, smooth muscle cells and adipocytes *in vitro* [7]. The cells are also able to form schwann cells which can be potentially used for injured spinal cord regeneration [7,11]. In the mouse, the pluripotency of embryonic stem (ES) cells is maintained by leukemia inhibitory factor (LIF), a member of IL-6 cytokine family, by preventing cell differentiation [12]. However, unlike murine ES cells, LIF is not necessary for maintaining pluripotency

in human ES cells [13]. In addition, LIF plays a crucial role for the development of preimplantation stage embryos *in vivo* [14]. In contrast to embryonic cells, myeloid cells and neuroblastoma cells were induced differentiation by LIF treatment [15,16]. Although LIF have been used to culture SKPs in human, pig and mouse [4,8,17], little is understood about the effect of LIF in SKPs. In this study, to evaluate the effect of LIF on the culture of SKPs *in vitro*, changes of the expression level of pluripotent stem cell- and neural crest-related genes in murine SKPs by LIF, a stemness maintaining factor, was investigated.

Materials and Methods

Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) and all liquid solutions were purchased from Invitrogen Korea (Seoul, Korea) unless otherwise stated.

Isolation and propagation of SKP

The murine SKPs were isolated by a previously described method with a few modifications [4,18]. To obtain skin from 5-6 weeks C57BL6 X DBA2 F1 mice, hairs of back were removed. After back skin was dissected and washed 4 times in phosphate-buffered saline with penicillin, skin pieces were minced into small pieces with blade. Small pieces of skin were transferred to a 100 mm petri dish containing 5 ml of 0.1% (w/v) trypsin solution and 5 ml of Hank's buffered salt solution with 2.4 mg/ml of dispase and 1 mg/ml collagenase type IV, and then, incubated for 60 min in 37°C, 5% CO_2 cell culture incubator. The incubated skin pieces were mixed by 30 times of pipetting using 10 ml glass pipette and the medium consisting of Dulbecco's modified Eagle medium and nutrient mixture F-12 (1:1, v/v; DMEM/F-12) was added. Skin cell suspension poured through a 100 and 40 μm Nylon cell strainer over 50 ml conical centrifuge tube. Dissociated cells were centrifuged at 1000 rpm for 5 min. After supernatant was removed, dissociated cells were re-suspended in 10 ml of DMEM/F-12 containing 2% B-27, 20 ng/ml bFGF and EGF (SKP medium). These cells were cultured in 25 cm^2 uncoated cell culture flasks in a 37°C, 5% CO_2 atmosphere (Fig. 1A). Fresh SKP medium was replaced every 2-3 days, and the cells started sphere forming at passage 1 (Fig. 1B).

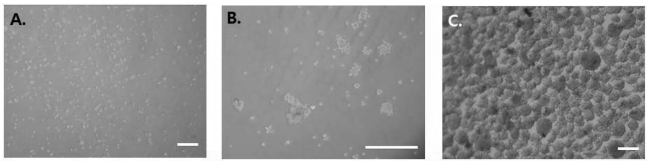

Fig. 1. Culture of murine skin precursor cells (SKPs). (A) Isolated single cells from murine back skin (passage 0). (B) Formation of primary spheres after 1 week of culture (passage 1). (C) Large spheres after 3 week of culture (passage 3). Bar, 40 μm.

Passaging of sphere-forming SKP

The medium containing suspending spheres was gently mixed by 10 ml glass pipette and moved to 15 ml conical tube. The spheres were centrifuged at 1000 rpm 5 min. After supernatant was removed, 1 ml of accutase was supplemented and the pellet was mechanically dissociated with a pipette and the clusters of cells with accutase were incubated for 3-5 min in a 37°C, 5% CO_2. The cells were t hen centrifuged at 1500 rpm for 3 min, and the pellet was mechanically dissected with a pipette. The single cells were cultured in 10 ml of SKP medium. The cells were passaged every 7 days. After the 2-3 weeks, proliferating cells formed spheres in suspension culture and size of the sphere increased with subcultural steps (Fig. 1C).

Adipogenic and neural differentiation for characterization of SKPs

To confirm the character of isolated cells from dermis as SKPs, adipogenic and neural differentiation were induced. For adipogenic differentiation, SKPs were attached on the bottom of 6-well plate containing SKP medium supplemented with 10% FBS and 10 ng/ml bFGF. When the cells grew to confluence, they were cultured in the same medium excluding bFGF for additional 5 days. For staining, differentiated cells were fixed with 10% formaldehyde in a 4℃ for 1 hr and then washed twice with PBS. The cells were then stained with Oil red O. For neural differentiation, SKPs were attached on laminin- and PDL-coated 6-well plate with neurobasal medium™ including B27 supplement and 0.5 mM dibutyryl cAMP for 14 day.

Treatment of LIF

After 21 day of culture (Passage 3), 1,000 IU murine LIF (Millipore, Billerica, MA, USA) was treated to SKPs in culture for 72 hr.

Total RNA extraction

Total RNA was isolated from sphere forming SKP using RNeasy™ Mini Kit (QIAGEN, Hilden, Germany). Briefly, the appropriate number of cells was pelleted by centrifugation and all supernatant was removed. The cells were disrupted by adding Buffer RLT Plus. The lysate was moved directly into a QIA shredder column, centrifuged for 2 min at 15,000 rpm to fully homogenize the lysate. Then the homogenized lysate was transferred to a gDNA eliminator spin column and centrifuged for 30 sec at ≥10,000 rpm. Ethanol (70%) was added to the flow-through and the sample including the precipitate was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 sec at ≥10,000 rpm and the flow-through was discarded. Buffer RW1 and buffer RPE were treated the same as ethanol. Then, buffer RPE was added again to the column and centrifuged for 2 min at ≥10,000 rpm and the flow-through was discarded. The RNeasy spin column was placed in a new 1.5 ml collection tube and RNase-free water added directly to the spin column membrane and centrifuged for 1 min at ≥10,000 rpm to elute the RNA.

First-strand cDNA synthesis

For the synthesis of first-strand cDNA by reverse transcriptase, reverse transcription was performed for 1 hr at 42°C in a final reaction volume of 25 μl containing purified total RNA, 5 μl of 5X reaction buffer (Promega, Madison, Wi, USA), 5 μl of dNTPs (each 2.5 mM), 2.5 μl of 10 mM synthesis primer, 0.5 μl of RNasin plus RNase inhibitor (40 U/ml; Promega), and 1 μl of M-MuLV reverse transcriptase (20 U/μl, Invitrogen).

Real time RT-PCR

For optimal quantification, primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The real time RT-PCR reaction was performed using the ABI PRISM 7500 system and SYBR Green PCR Master Mix (Applied Biosystems). All points of the standard curve and all samples were run in triplets as technical replicates. The standard curves were calculated using the verified DNA as template for murine *GAPDH*. In each run 1 μl of cDNA was used as template and the sample was added to 5 μl double-distilled water, 2 μl of forward and reverse primers (20 pmol/ml) and 10 μl SYBR Green PCR Master Mix. The following amplification procedure was employed: denaturation stage (95°C for 10 min), amplification and quantification stage repeated 40 times (94°C for 15 sec, 60°C for 1 min with single fluorescence measurement), dissociation curve stage (temperature increments of 0.1°C per 30 sec starting from 60 to 95°C with fluorescence measurement). Data was analyzed with 7500 System Sequence Detection software (Applied Biosystems), which for all samples calculated that starting quantities of all candidate reference genes, based on the standard curves for these genes.

Statistical analysis

Each experiment was replicated three to five times. Mean gene expression values were analyzed by *t*-test to compare parameters between the different study groups. The interaction between replicate and treatment was also tested using two-way ANOVA. Difference at $P < 0.05$ was considered significant.

Results

Characterization of SKPs by *in vitro* differentiation

The character SKPs were confirmed by neural and adipogenic differentiation. The murine SKPs which attached on the laminin and PDL coated plate in neural differentiation medium were differentiated into neural cells after 14 day of culture. Differentiated cells from SKPs showed heterogeneous population of various types of neural cells and schwann cells. The cells of adipogenic differentiation induction showed morphology of adipocytes and this was confirmed by Oil red O staining (Fig. 2).

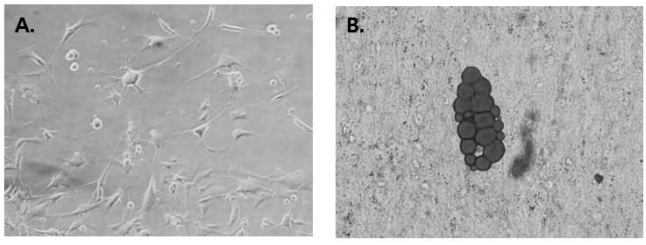

Fig. 2. *In vitro* differentiation of SKPs. (A) Heterogeneous neural-like progeny derived from SKPs. (B) Adipogenic cells stained with Oil Red O.

Expression of pluripotency and neural crest marker genes

The expression of *Oct4* significantly increased by LIF treatment whereas the expression of *Nanog* and *Ngfr* decreased by the treatment ($P < 0.05$). There was no significant change

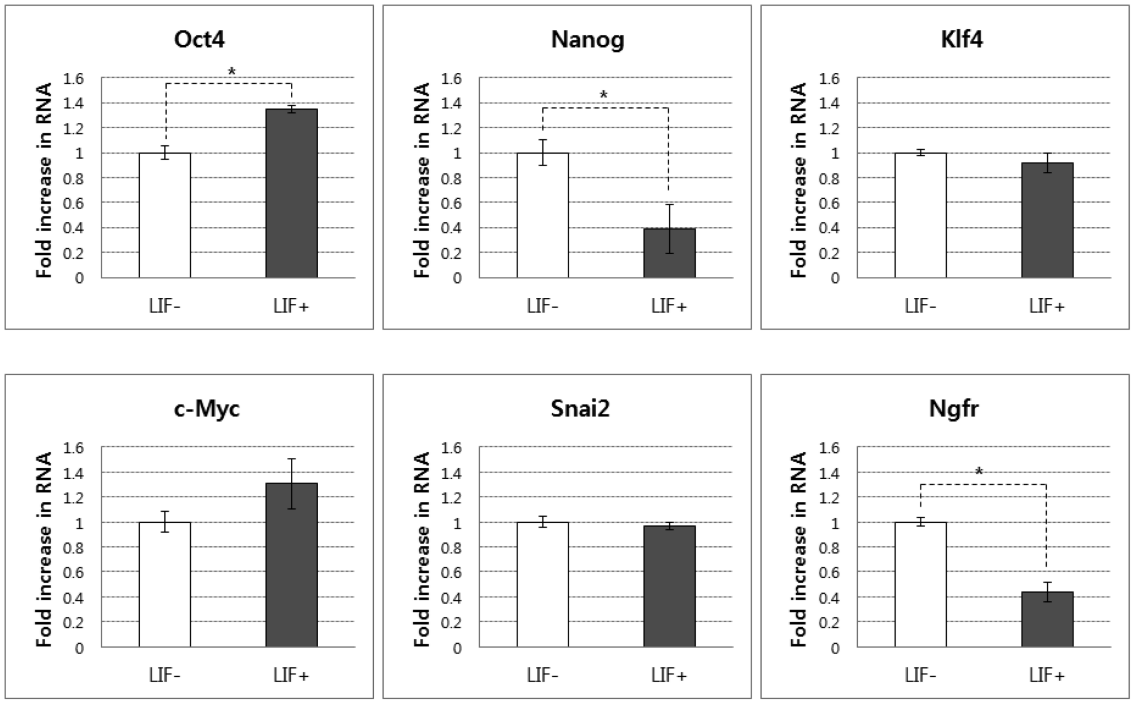

Fig. 3. Gene expression analyses in murine SKPs after LIF (1,000 IU) treatment. Quantitative real-time PCR for genes of pluripotent stem cells (*Oct4, Nanog, Klf4* and *c-Myc*) and neural crest cells (*Snai2* and *Ngfr*). All values were depicted by the ratio to the expression in the control group (values in SKPs without LIF treatment = 1). Data are expressed as mean ± SD (n = 4). *P < 0.05.

on the expression level of *c-Myc, Klf4* and *Snai2*; Fig. 3).

Discussion

SKPs which are able to form sphere in suspension culture can differentiate to various lineage progeny [4]. In the previous study, we showed that valproic acid, histone deacetylation inhibitor, enhances the expression level of neural crest related genes, whereas reduces the expression level of pluripotency-related genes in murine SKPs [18]. In this study, the change of gene expression was observed after LIF treatment. LIF is a critical factor for maintaining the pluripotency of murine ES cells by inhibiting cell differentiation through JAK/STAT pathway [13,19,20]. The JAK/STAT signaling pathway is essential for self-renewal of stem cells [13]. When the ES cells are maintained undifferentiated state by LIF, they are expressed pluripotency-related genes such as *Oct4* and *Nanog* [19,21-23]. LIF signaling also significantly enhance the STAT3 expression in porcine SKPs [24]. As spheres of SKPs include progeny of neural crest cells, the cells expressed neural crest-related genes such as *Snai2* and *Ngfr* [4,7,8]. In this study, LIF increased the expression level of *Oct4* which is POU domain transcription factor and sustain self-renewal and pluripotency. This shows that LIF enhances stemness of SKPs. However, expression level of *Nanog* was decreased by LIF treatment. Although Nanog is important for maintaining pluripotency in ES cells, it also accelerates oesteogenic lineage differentiation in the human mesenchymal cells [25,26]. In the present experiment, *Ngfr*, neural crest cell marker, was decreased by LIF and the result represents that the potential of neural crest lineage differentiation was suppressed following LIF treatment. However, *Snai2* expression was unchanged by LIF treatment. There is a report claiming that the *STAT3* expression was increased by LIF treatment in porcine SKPs, whereas the same treatment did not change the level of *Snai2* expression [24], and the data of the present study also imply that LIF signaling does not affect *Snai2* expression in the murine SKPs. Enhanced *Oct4* expression may keep pluri- or multi-potency by alleviating differentiation potential in murine SKPs. The previous study showed that cell proliferation of human SKPs was decreased by LIF [17]. In the present study, decreased expression of *Nanog* following LIF treatment may also reduce proliferative capacity of murine SKPs because Nanog is a crucial factor for cell proliferation in adult stem cells [21,23].

In conclusion, the finding implicates that LIF may support maintenance of stemness in SKPs by suppressing lineage differentiation and partly by controlling cell proliferation

and this may contribute to controlling stemness of SKPs and other stem cells. In addition, The LIF treatment resulting stemness elevation can be a tool for efficient production of pluripotent stem cells using SKPs which can be collected non-invasively from the patient.

Acknowledgments

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