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Leukemia inhibitory factor induces multi-lineage differentiation of adult stem-like cells in kidney via kidney-specific cadherin 16

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Abstract

Side population (SP) is reported to be a stem cell-rich population. In the presence of leukemia inhibitory factor (LIF), cultured kidney SP cells differentiated into multi-lineage in collagen gel but not in synthesized polymer that has no cell adhesion factor. In cultured kidney SP cells, gene expression of kidney-specific cadherin 16 was specifically upregulated in collagen gel but not in synthesized polymer. Moreover, decreasing cadherin 16 expression using siRNA abolished LIF-induced multi-lineage differentiation of kidney SP in collagen gel. These results indicated that LIF induced multi-lineage differentiation of adult stem-like cells in kidney via cadherin 16.

Keywords: Stem cell; Regenerative medicine; Cadherin 16

Existence of adult stem cells is reported in various kinds of tissues, and the so-called side population (SP) is reported to be a stem cell-rich population [1–4]. SP cells exist in various kinds of tissues [5], but the mechanism of multi-lineage differentiation has not been clarified for each type of tissue-derived SP cell, especially in the kidney [6]. In this study, we cultured kidney SP cells both in type I collagen gel and thermoreversible gelation polymer (TGP) that has no cell adhesion factor, and compared differentiation of the cells. Leukemia inhibitory factor (LIF) induced multi-lineage differentiation of kidney SP cells in collagen gel but not in TGP. To clarify the key adhesion factor that determined LIF-induced multi-lineage differentiation of kidney SP cells, DNA microarray analysis was performed. Microarray analysis clarified that kidney-specific cadherin 16 [7] was specifically upregulated in collagen gel but not in TGP. Finally, we examined the role of cadherin 16 in LIF-induced multi-lineage differentiation of kidney SP cells by decreasing cadherin 16 expression using siRNA. Our results showed that cell adhesion factor such as cadherin 16 is required to induce multi-lineage differentiation of adult stem-like cells such as SP cells in the kidney.

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Materials and methods

SP cell sorting and culture. C57/B6 mice (8 weeks of age) were purchased from Clea Japan (Tokyo, Japan). All the procedures described here were approved by the Animal Committee of the University of Tokyo. Mice were anesthetized and perfused via the abdominal aorta with normal saline. The kidneys were harvested and the tissue was minced with a razor blade and digested with collagenase. The cell suspensions were filtered through a cell-strainer (Falcon 2350) to remove debris. The filtrates were analyzed as previously described [4]. Briefly, reserpine was added at a final concentration of 50 μM [4], and cells were then incubated at 37 °C for 15 min and Hoechst dye was added. Kidney SP cells were isolated using a FACS Vantage (BD Biosciences) for flow cytometric sorting. After FACS sorting, kidney SP cells were cultured on MEF feeder cells (RPMEF-N: Dainippon Pharmaceutical, Osaka, Japan) for 7 days with RES101 medium (Dainippon Pharmaceutical, Osaka, Japan) containing leukemia inhibitory factor (LIF: 10 ng/ml). After pre-culture on MEF feeder cells for 7 days, the kidney SP cells were re-seeded in type I collagen gel (Koken, Tokyo, Japan) or thermoreversible gelation polymer (TGP) with RES101 medium until day 28. TGP was purchased from Mebiol (Tokyo, Japan). In Figs. 2B–D, cells just after FACS sorting were cultured in collagen gel or TGP gel for 48 h in the presence or absence of LIF.

Microarray and real-time PCR analysis. DNA microarray hybridization experiments were performed using Clontech Atlas glass Mouse 3.8 I microarray (BD Biosciences) according to the manufacturer's protocol. The protocol and a complete list of genes can be viewed at http://www.bdbiosciences.com/clontech/techinfo/manuals/index.shtml. The DNA arrays were scanned using GenePix4000A [8]. Quantitative real-time PCR was performed using commercially available TaqMan probes (AQP2: Mm00437575 m1, CD31: Mm00476702 m1, neurofilament: Mm00456201 m1, and GATA4: Mm00464689 m1), and analyzed on an ABI PRISM 7000 sequence detector system (BD Biosciences). Quantitative values were obtained from the threshold PCR cycle number at which an increase in signal associated with exponential growth of the PCR product started to be detected. The relative mRNA levels in each sample were normalized to its GAPDH content.

RNA interference. The siRNA for cadherin 16 (pre-designed siRNA, ID#60533) was purchased from Ambion. Transfection was carried out using Qiagen RNAi Starter Kit. 14.5 ml of 20 mM siRNA and 33.75 ml of transfection reagent were added to a medium in a total volume of 250 ml, and left for 15 min at room temperature to allow the formation of transfection complexes. Subsequently, the mixture was diluted with a type I collagen gel to a total volume of 1 ml, and the FACS sorted kidney SP cells were incubated in the gel with LIF-containing culture medium for 48 h.

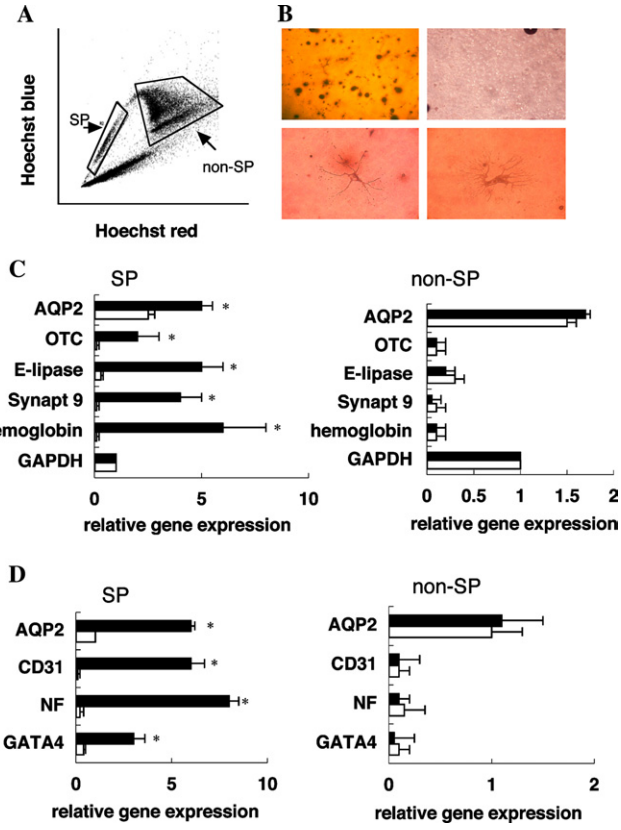

Fig. 1. LIF-induced multi-lineage differentiation of kidney SP cells in collagen gel. (A) Representative FACS profile of SP cells and non-SP cells isolated from the kidney. (B) Representative photographs of cultured kidney SP cells in collagen gel (left upper and lower panels) and TGP gel (right upper) on day 28. Upper panels are low power (40×: phase contrast) and others are high power (400×: phase contrast) photographs. (C) Results of microarray analysis of several lineage-specific genes in cultured kidney SP cells and non-SP cells on day 28 (black bars) compared to day 7 (white bars). Hemoglobin: hemoglobin β-chain, Synapt 9: synaptotagmin, E-lipase: endothelial lipase, OTC: ornithine decarbamylase, and AQP2: aquaporin 2. Expression was normalized to that of GAPDH. Values represent means ± SEM. $*p < 0.05$ vs. day 7. (D) Results of quantitative PCR of representative genes of three germ layers (ectoderm: NF; mesoderm: CD31, AQP2; and endoderm: GATA4). Closed bars are day 28 and open bars are day 7. Values represent means ± SEM ($n = 4$). $*p < 0.05$ vs. day 7.

Results

LIF-induced multi-lineage differentiation of kidney SP cells in collagen gel

We isolated whole kidney cells from C57/B6 mice and stained them with Hoechst 33342 dye [9]. The isolated cells were subjected to FACS analysis and immediately used for cell culture (Fig. 1A). Kidney SP cells attached and formed colonies on a mouse MEF feeder layer, and the colonies increased in size until around day 7 but for some reason stopped growing, and then we re-seeded the cells in type I collagen or TGP gel on day 8. On day 28, kidney SP cells formed island-like colonies and tube-like structures in collagen gel (Fig. 1B, left upper panel and lower panels). On the other hand, kidney SP cells never formed such structures in TGP gel not containing cell adhesion factor (Fig. 1B, upper right panel). To examine the multi-lineage differentiation of kidney SP cell in collagen gel and TGP gel, we compared the gene expression of culture SP cells on day 28 with that on day 7 by microarray analysis. Gene expression of several lineage-specific genes such as hemoglobin β-chain [10] (erythrocyte), endothelial lipase [11] (endothelial cell), synaptotagmin [12] (neuron), and ornithine decarbamylase [13] (liver) was very low on day 7, but was significantly upregulated in collagen gel culture on day 28 (Fig. 1C). On the other hand, these genes showed no change in TGP gel culture (Fig. 1C). To examine the

differentiation of kidney SP cells into three germ layers, we performed quantitative real-time PCR analysis of representative genes of the three germ layers (ectoderm: neurofilament (NF); mesoderm: CD31, aquaporin 2 (AQP2); and endoderm: GATA4). In collagen gel culture on day 28, all genes were significantly upregulated as compared to day 7 (Fig. 1D). As in the case with microarray analysis, there was no significant change in gene expression of these genes in TGP gel culture (Fig. 1D).

LIF-induced multi-lineage differentiation was mediated via cadherin 16

To clarify the mechanism of LIF-induced multi-lineage differentiation of kidney SP cells in collagen gel, but not in TGP gel, we tried to clarify the contribution of cell adhesion factors such as cadherins. Compared to TGP gel culture, cadherin 16 was specifically upregulated in collagen gel with LIF on day 28 (Fig. 2A). Next, we examined the effect of LIF on cadherin 16 expression in cultured kidney SP cells by real-time PCR. Treatment with LIF significantly upregulated cadherin 16 in kidney SP cells cultured in collagen gel but not in TGP gel (Fig. 2B). On the other hand, treatment with LIF showed no effect on kidney non-SP cells (Fig. 2B). Finally, we examined whether LIF-induced multi-lineage differentiation is mediated by cadherin 16, we treated kidney SP cells and non-SP cells with siRNA for cadherin 16 and cultured the cells in collagen gel in the presence of LIF. As shown in Fig. 2C, LIF-induced multi-lineage differentiation was significantly inhibited by pre-treatment with siRNA in SP cells. In non-SP cells, LIF alone showed minimum effect and siRNA showed no significant effect (Fig. 2C).

Discussion

Our present results demonstrated that LIF induced multi-lineage differentiation of adult stem-like cells such as SP cells in the kidney via kidney-specific cadherin 16. Cadherin 16 is a unique, tissue-specific member of the cadherin family of cell adhesion proteins that is expressed in the adult kidney and developing genitourinary tract [7,14]. During embryonic development, cadherin 16 is expressed in developing renal tubules in the metanephros and the expression of cadherin 16 is developmentally regulated as well as tissue-specific. Recently, a gene that is homologous to cadherin 16 has been identified in zebrafish, and several studies suggested that cadherin 16 may function as a cell adhesion protein that is required for maintaining tubular integrity [15]. Moreover, Yoshino et al. [16] reported that leukemia inhibitory factor (LIF) is upregulated in ARF, and is considered to play a role for regeneration process. Although the precise functional role of cadherin 16 remains to be determined, our results strongly suggest that cadherin 16 may play an important role in LIF-induced regenerative processes as in developing processes.

TGP gel is a chemically synthesized hydrophilic 3D culture material, which is composed of poly(*N*-isopropylacrylamide-co-*n*-butyl methacrylate) and polyethylene glycol (PEG), and acts as a hydrophilic 3D scaffold without adhesion factors. Recently, we have found that osteogenic differentiation of human mesen-

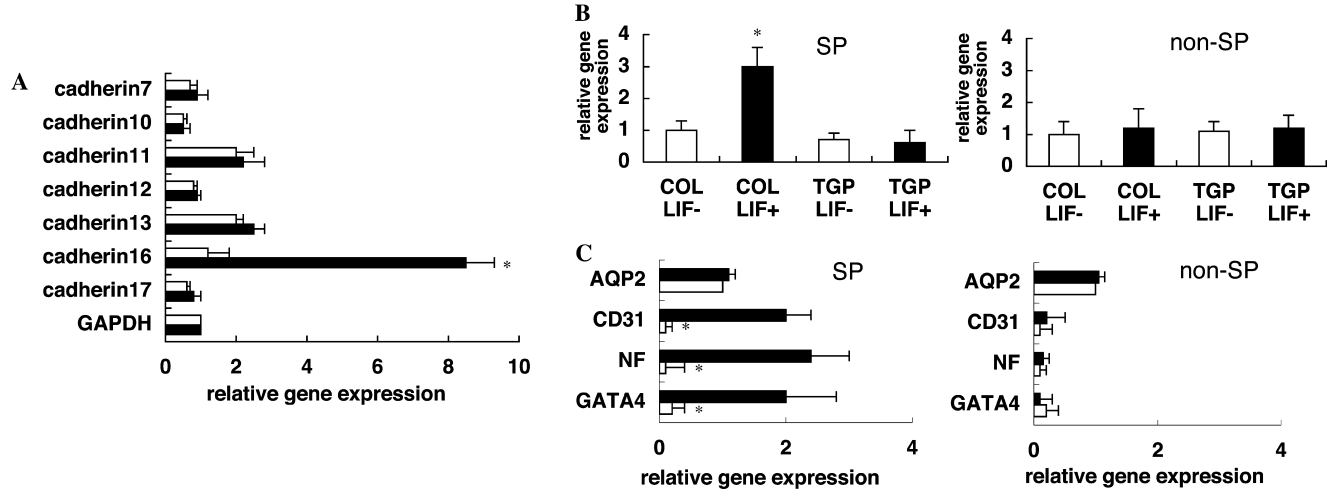

Fig. 2. (A) Microarray analysis of cadherins in cultured kidney SP cells in collagen gel (closed bars) and TGP gel (open bars) on day 28. Average expression levels of each gene were calculated from three independent hybridizations. Expression was normalized to that of GAPDH. Values represent means ± SEM. *$p < 0.05$ vs. TGP gel. (B) Results of quantitative PCR analysis of cadherin 16 in kidney SP cells and non-SP cells cultured in collagen gel or TGP gel. Values represent means ± SEM ($n = 4$). *$p < 0.05$ vs. COL LIF. (C) Results of quantitative PCR analysis of representative genes of three germ layers (ectoderm: NF; mesoderm: CD31, AQP2; and endoderm: GATA4). Closed bars are LIF alone and open bars are LIF and siRNA. Values represent means ± SEM ($n = 4$). *$p < 0.05$ vs. LIF alone.

chymal stem cells (HMSC) was augmented in TGP culture but not in collagen gel [17]. Compared to bone marrow, kidney tissue is rich in extracellular matrix, and this may explain why kidney SP cells required adhesion factors such as cadherin 16 for multi-lineage differentiation. The structure of TGP makes it possible to vary the diffusive speed according to the character of the molecules [17]. TGP may be able to maintain a high local concentration of hydrophobic molecules and hydrophilic large molecules, and this creates a concentration gradient of each molecule. Our results showed that cadherin 16 played key roles in LIF-induced multi-lineage differentiation but it may be possible that TGP may keep high local concentration of LIF-induced unknown factors that can promote multi-lineage differentiation.

Although the physiological function of cadherin 16 is still unclear, the promoter of the mouse cadherin 16 is recently clarified [14,18]. The promoter is TATA-less but contains other consensus promoter elements including an initiator element, GC boxes, and a variant CAAT box as well as potential binding sites for activator protein (AP)-2, hepatocyte nuclear factor (HNF)-1, HNF-3, basic helix–loop–helix (bHLH) proteins, and GATA factors. LIF is a member of the interleukin 6 (IL-6) family and IL-6 induces AP-2 in kidney mesangial cells [19]. These results suggest that LIF may induce cadherin 16 via induction of AP-2 in cultured kidney SP cells in collagen gel.

In summary, our results demonstrated that LIF induced multi-lineage differentiation of adult stem-like cells such as SP cells in the kidney via kidney-specific cadherin 16. These results suggest a new functional role of kidney-specific cadherin 16 in regenerative processes of kidney disease.

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