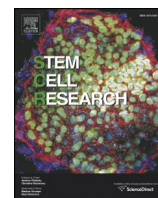


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Behavior of leucine-rich repeat-containing G-protein coupled receptor 5-expressing cells in the reprogramming process



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ABSTRACT

It remains unclear what cells are proper for the generation of induced pluripotent stem cells (iPSCs). Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) is well known as a tissue stem cell and progenitor marker, both of which are reported to be sensitive to reprogramming. In the present study, we examined the reprogramming behavior of Lgr5-expressing cells (Lgr5+ cells). First, we compared reprogramming behavior using mouse Lgr5+ and Lgr5 negative (Lgr5−) hair follicles (HFs). The number of alkaline phosphatase staining-positive cells was lesser in a well of Lgr5+ HFs than in Lgr5− HFs; however, the ratio of Nanog+SSEA1+ cells in the cell mixture derived from Lgr5+ HFs was much higher than that from Lgr5− HFs. Lgr5+ cells could be induced from mouse embryonic fibroblasts (MEFs) after transduction with Yamanaka factors. As shown in HFs, the progeny of Lgr5+ cells arising from MEFs highly converted into Nanog+ cells and did not form Nanog− colonies. The progeny represented the status of the late reprogramming phase to a higher degree than the nonprogeny. We also confirmed this using human Lgr5+ cells. Our findings suggest that the use of Lgr5+ cells will minimize sorting efforts for obtaining superior iPSCs.

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1. Introduction

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the transduction of four embryonic transcriptional factors (Takahashi et al., 2007b; Takahashi and Yamanaka, 2006; Yu et al., 2007). This discovery provides not only a novel opportunity for regenerative medicine but also innovative cell therapies specific for patients or diseases (Romano et al., 2014; Sinnecker et al., 2014). However, many issues regarding iPSCs remain. One of these is their low generation efficiency. After the reprogramming induction, most cells are converted into partially reprogrammed cells (Utikal et al., 2009). Recently, some studies showed that more primitive cells such as neural stem cells are more readily converted into iPSCs than terminally differentiated cells because of their original expression of genes that are related to cell reprogramming (Kim et al., 2008; Kleger et al.,

2012; Tsai et al., 2011). On the other hand, it has been reported that progenitors that are more readily differentiated than stem cells can effectively be reprogrammed into iPSCs (Guo et al., 2014). The types of cells that are sensitive to reprogramming remain controversial.

Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) was first reported to be a tissue stem cell marker, e.g., related to the small intestine and colon (Barker et al., 2008, 2007; Haegebarth and Clevers, 2009; Jaks et al., 2008). Many studies have recently found that Lgr5 is a marker of progenitors and not only tissue stem cells (Ng et al., 2014; Ren et al., 2014; Yee et al., 2013), both of which are reported to be sensitive to successful reprogramming. However, the significance of Lgr5-expressing cells (Lgr5+ cells) for the generation of iPSCs remains unexamined.

In the present study, we evaluated the reprogramming behavior of Lgr5+ cells. Here we showed that the use of mouse Lgr5+ hair follicles resulted in the induction of less alkaline phosphatase staining-positive (AP+) cells but a greater number of Nanog-positive (Nanog+) cells than the use of Lgr5-negative (Lgr5−) cells. In addition, Lgr5+ cells emerged after reprogramming induction in mouse embryonic fibroblasts (MEFs) and normal human dermal fibroblasts

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(NHDFs), and both showed the strong advantage of the conversion into Nanog⁺ cells.

2. Materials and methods

2.1. Cells

For the isolation of hair follicles (HFs), we prepared epidermal HFs from neonatal (0–2 days old) Lgr5-EGFP-IRES-CreERT2/Rosa26-loxp-stop-loxp-LacZ mice (Lgr5-KI mice) following the method of a previous study (Lichti et al., 2008) with some minor modifications. In brief, after the removal of the whole skin of newborn mice, the skins were placed in 0.25% trypsin at 4 °C for over 16 h to isolate the epidermis and dermis. The isolated epidermis was minced into small pieces using a scalpel. HFs were isolated from the minced epidermis based on weight, as HFs are heavier than other epidermal cells. We prepared MEFs from E14.5 of C57BL/6 wild-type (Lgr5-WT) mice and Lgr5-KI mice according to a previous study (Takahashi et al., 2007a). The culture medium used for MEFs was embryonic fibroblast (EF) medium: Dulbecco's modified Eagle's medium (DMEM) (SIGMA D5796) supplemented with 10% fetal calf serum (FCS), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen 15140-122). Embryonic stem cell (ESC) medium was used during mouse cell reprogramming inductions: knockout DMEM (GIBCO 10829-018) supplemented with 15% FCS, 2 mM L-glutamine (GIBCO 25030-081), 0.1 mM nonessential amino acids (GIBCO 11140-050), hLIF (WAKO 125-05603), 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Images were acquired using BZ-9000 (KEYENCE). All procedures were conducted in accordance with the guidelines for the Care and Use of Animals established by the Animal Care and Use Committee of Gifu University. NHDFs (Takara D10051) were cultured in EF medium. For NHDF reprogramming, the hiPSC medium was used: DMEM/F12 supplemented with 20% KSR (GIBCO 10828028), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 2-mercaptoethanol, penicillin and streptomycin and bFGF (WAKO 064-04541). For the feeder-free culture, MEF-conditioned medium was used; hiPSC medium was exposed to MEF-feeder cells overnight.

2.2. Reprogramming induction

For reprogramming induction of mouse cells, pMx-based retroviral vectors encoding mouse *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* were used. Their retroviruses were produced as previously described (Takahashi et al., 2007a). For HFs, sorted HFs were plated onto feeder cells in 12-well plates with low calcium medium: S-MEM (GIBCO 11380-037) containing 8% FCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen). After 2 days, viruses with 4 µg/mL of polybrene were added to the cells. To enhance the efficiency of infection, we performed spinfection at 500 × g for 1 h for 2 consecutive days. On the day following the second spinfection, the medium was replaced with ESC medium. Approximately 10 days after the first infection, cells were reseeded onto feeder cells at 5 × 10⁴ cells per well of 12-well plates. The reprogramming of MEFs was induced as previously described (Takahashi et al., 2007a; Takahashi and Yamanaka, 2006). NHDFs reprogramming was induced using episomal vector as previously described (Okita et al., 2011). All procedures were conducted in accordance with the guidelines for recombinant DNA research established by Gifu University.

2.3. Flow cytometry

Cells were dissociated by incubation in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA), followed by pipetting to obtain single-cell suspensions. To detect Nanog expression, cells were fixed with 4% paraformaldehyde (PFA) for 10 min at 37 °C, followed by permeabilization with cold 90% methanol for 20 min on ice. After being blocked with 0.5% bovine serum albumin (BSA) for 10 min, the cells

were incubated with rabbit anti-mouse Nanog antibody (Cell Signaling Technology, #8822) for 60 min at room temperature, followed by Alexa Flour-conjugated secondary antibody (Abcam) for 30 min. In addition, Alexa Flour 647-conjugated mouse anti-SSEA-1 antibody (Santa Cruz, sc-21702 AF647) was added before analysis. Cells were directly carried over into the analysis after the dissociation to detect and sort Lgr5-EGFP. For cell sorting by LacZ activity, they were stained using the FluoReporter LacZ Flow Cytometry Kit (Molecular Probes) according to the manufacturer's instructions. In the case of human cells, Lgr5 positive cells were sorted using anti-human Lgr5 PE-conjugated antibody (R&D Systems). A cutoff was set using unstained cells. Cells were analyzed on FACSCanto, FACSAria (BD Biosciences) or FlowJo.

2.4. Alkaline phosphatase (AP) staining

AP staining was performed by following a RIKEN protocol. Cells were washed with AP buffer (0.1 M NaCl, 0.1 M Tris, 0.05 M MgCl₂·6H₂O, pH 9.5) and then stained with the AP substrate: BCIP and NBT diluted in AP buffer for 30 min. Stained cells were fixed in 4% PFA at 4 °C for 30 min. The evaluation of AP staining positive (AP⁺) cells was conducted using the software Image J.

2.5. RNA extraction, quantitative polymerase chain reaction (qPCR), and microarray analysis

Total RNA was extracted from cells using ISOGEN (Nippon GENE). Reverse transcription (RT) was performed using the High-Capacity cDNA Transcription Kit (Applied Biosystems). Gene expression was analyzed on LC 480 (Roche) or 7900HT (Applied Biosystems) using the KAPA SYBR Fast qPCR Kit (KAPA BIOSYSTEMS). Primers for RT-qPCR used in the present study are shown in Table S1. Microarray analysis was performed using the Mouse Gene 1.0 ST Array (Affymetrix) in accordance with the manufacturer's instructions. All data analyses were performed using the GeneSpring GX software program (version 12; Agilent Technology).

2.6. X-gal staining

MEFs were cultured in ESC medium with 1 µM 4-hydroxytamoxifen (4-OHT) (SIGMA) from day 5 after retroviral infection. X-gal staining was performed according to the manufacturer's instructions (Takara, Z1780N) on day 14 after reprogramming induction.

2.7. Embryoid body (EB) formation and in vitro differentiation

For EB formation, dissociated cells were cultured in DMEM/F12 containing 5% KSR, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 2-mercaptoethanol, Y-27632 (WAKO 036-24023), penicillin and streptomycin. Seven days after floating culture, EBs were transferred to gelatin-coated plates and cultured in DMEM containing 10% FBS for another 7 days to induce spontaneous differentiation.

2.8. Immunocytochemistry

Cells were fixed with 4% PFA for 15 min at room temperature, followed by permeabilization and blocking using blocking buffer [1% BSA, 0.3% TritonX-100 in phosphate-buffered saline (PBS)] for 60 min. The cells were incubated with each primary antibody overnight at 4 °C, followed by Alexa Flour-conjugated secondary antibodies (Life Technologies) for 60 min. After washing with PBS, the samples were mounted using Prolong Gold Antifade Reagent (Life Technologies), followed by overnight curing on a flat surface in the dark at 4 °C. To obtain the overall picture in each well, multiple connected pictures composed of 4 × 9 images were created using BZ-9000 (KEYENCE) (Fig. 4E). The primary antibodies used were as follows: rabbit anti-mouse Nanog antibody (Cell Signaling Technology, #8822), rabbit anti-

human NANOG antibody (Abcam, ab21624), mouse anti-human TRA-1-60 antibody (Abcam, ab16288), SOX17 (R&D Systems, AF1924), α SMA (SIGMA, A2547), and Tuj1 (SIGMA, T8660).

2.9. Overexpression and knockdown experiment

Mouse *Lgr5* (NM_010195.2) was cloned from mouse liver by PCR amplification with KAPA HiFi DNA polymerase (KAPA BIOSYSTEMS), and *Lgr5* cDNA was subcloned into pMXs retrovirus vector. Virus encoding *Lgr5* was obtained as well as Yamanaka factors. For overexpression of *Lgr5*, MEFs were incubated with a virus encoding *Lgr5* concurrently or 7 days after reprogramming induction. A virus encoding empty vector was used as control. For the knockdown experiment, 25 bN siRNAs (*Lgr5*#1 Mm_010195.2, cont Mission_SIC-001, SIGMA) were transfected with Mission siRNA Transfection (SIGMA) at day 9 after reprogramming induction. Primers for evaluating the efficiency of knockdown were as follows: *Lgr5* fw-GGAAATGCTTTGACACACATTC, rv-GGAAGTCATCAAGGTTATTATAA.

2.10. Statistical analyses

Values were expressed as means \pm standard deviations (SDs). Statistically significant differences ($p < 0.05$) were determined using the Student's *t*-test between two groups or using one-way ANOVA, followed by a Tukey's test post hoc test among three groups.

3. Results

3.1. Conversion into Nanog + cells was promoted in mouse *Lgr5* + HF

Lgr5 is known as a marker of progenitors and tissue stem cells, both of which are reported to be sensitive to reprogramming. We first investigated the reprogramming behavior of *Lgr5* + cells. Because of their ease of application, we used HF as a source of *Lgr5* + cells. *Lgr5* + HF were sorted using *Lgr5*-KI neonatal mice, as shown in Fig. 1A. As a control, *Lgr5* – and all HF (indicated as “All”) were additionally sorted using a flow cytometer.

We performed cell reprogramming of each group using Yamanaka factors (Fig. 1B). The number of reseeded cells was equivalent to 50,000 cells in each group. Fourteen days after reprogramming induction, much more AP + cells were detected in *Lgr5* – and All HF wells than in *Lgr5* + HF wells (Fig. 1C). AP + is one of the reprogramming initiation phase markers (Brambrink et al., 2008; David and Polo, 2014). AP + cells in *Lgr5* – HF and All HF wells did not form iPSC-like colonies, whereas almost all AP + cells in *Lgr5* + HF wells formed iPSC-like colonies (Fig. 1C). To exclude the possibility that AP + cells may be detected in *Lgr5* + HF without reprogramming induction, we performed AP staining using *Lgr5* +, *Lgr5* –, and All HF 14 days after sorting without reprogramming induction. No positive cells were detected in each group (Fig. 1D), thereby confirming that the observed AP + cells in Fig. 1C were the result of reprogramming induction.

Next, we evaluated the Nanog and SSEA1 expression of cells derived from *Lgr5* +, *Lgr5* –, and All HF at 14 days after reprogramming induction using fluorescence-activated cell sorting (FACS) analysis. Nanog and SSEA1 are markers of maturation and initiation-maturation phase of reprogramming, respectively (Brambrink et al., 2008; David and Polo, 2014). A much larger proportion of Nanog + SSEA1 + cells was detected in the cell mixture from *Lgr5* + HF (72.9 \pm 11.7%) than in others (*Lgr5* – HF: 15.5 \pm 14.7%, All HF: 28.7 \pm 12.5%) (Fig. 1E and F). We additionally examined Nanog + SSEA1 + expression using mouse ES cells as a control. Under the same condition, ES cells had 63.7% Nanog + SSEA1 + cells (Fig. 1E). Considering these results, the observed ratio in cells from *Lgr5* + HF was comparable to or greater than that in ES cells. On the other hand, cells from *Lgr5* – and All HF showed a higher ratio of Nanog – SSEA1 + cells than those from *Lgr5* + HF (Fig. 1E

and G). These findings suggest that reprogramming maturation is more likely to be occurred in *Lgr5* + HF than *Lgr5* – HF.

Previous studies reported that cells that are sensitive to reprogramming originally express the genes necessary for reprogramming (Kim et al., 2008; Tsai et al., 2011). Thus, we evaluated several gene expressions in *Lgr5* +, *Lgr5* –, and All HF before reprogramming induction via qPCR analysis for comparison with those in MEFs. Among Yamanaka factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*), the levels of *Oct3/4* and *Sox2* expression in *Lgr5* + HF were low (Fig. S1A and B), and the levels of *Klf4* and *c-Myc* expression in *Lgr5* + HF were similar to those in All HF (Fig. S1C and D). E-cadherin and N-cadherin, encoded by the genes *Cdh1* and *Cdh2*, respectively, showed similar expression levels among the three groups (Fig. S1E and F). With regard to *Nanog*, *Lgr5* – HF showed higher expression levels than *Lgr5* + HF (Fig. S1G).

3.2. *Lgr5* + cells emerged by Yamanaka factors transductions in MEF

Lgr5 + HF were effectively converted into Nanog + cells. To investigate whether this is specific to HF, we investigated the expression of *Lgr5* during MEF reprogramming. We found that the level of *Lgr5* mRNA in bulk MEFs was elevated from 10 days after reprogramming induction (Fig. 2A). In agreement with this, although it was not detected in MEFs (day 0), the *Lgr5*-EGFP signal could be more frequently detected in *Lgr5*-KI mice 14 days after reprogramming induction using a flow cytometer (WT: 0.8%, KI: 8.2%), as observed when using fluorescence microscopy (Fig. 2B–C and Fig. S2A). The slightly detected EGFP signal in *Lgr5*-WT mice appeared to be a nonspecific signal as they did not contain EGFP reporter. Considering that the expression of *Lgr5* from iPSCs is minimal (Fig. S2B) (Ohnishi et al., 2014), it is suggested that *Lgr5* expression is transiently induced by the transduction of Yamanaka factors.

For analysis of the detected *Lgr5* + cells derived from MEFs, we sorted *Lgr5* + (M-*Lgr5* +) and *Lgr5* – (M-*Lgr5* –) cells using MEFs derived from *Lgr5*-KI mice transduced with Yamanaka factors 14 days after reprogramming induction, as shown in Fig. 2B, followed by seeding at a density of 1500 cells/well onto 24-well plates with irradiated feeder cells. One more week later (21 days after reprogramming inductions), growing colonies were frequently detected in wells seeded with M-*Lgr5* – cells (Fig. 3A left). In contrast, almost no colonies could be detected in wells seeded with M-*Lgr5* + cells (Fig. 3A right). AP staining confirmed this (Fig. 3B and C).

To examine the reprogramming status of M-*Lgr5* + cells, microarray analysis was performed using M-*Lgr5* + cells in addition to the original MEFs, iPSCs, and ESCs as comparison subjects. M-*Lgr5* + cells, which emerged 14 days after reprogramming induction, expressed both epithelial and mesenchymal genes, which differed from both MEFs and iPSCs (ESCs) (Fig. 3D). In addition, the expression of genes involved in Wnt signaling was examined. The expression levels of *Lef1* and *Hnf1a* (also known as *Tcf1*) were downregulated and that of *Tcf4* was upregulated in M-*Lgr5* + cells compared with pluripotent stem cells (Fig. 3E). Previous studies reported that cell proliferation rate has an effect on cell reprogramming (Guo et al., 2014; Gupta et al., 2015; Xu et al., 2013). Although the proliferation of M-*Lgr5* + cells without forming colonies could be observed as shown in Fig. 3A right panel, we found that the expression levels of *Ccne1* and *Ccnb1* in M-*Lgr5* + cells were lower than those in iPSC, ESCs and even original MEFs (Fig. 3F).

3.3. The number of AP + cells was smaller but that of Nanog + cells was larger in progeny of M-*Lgr5* + cells than nonprogeny

Considering that iPSCs rarely express *Lgr5*, M-*Lgr5* – cells which were detected at 14 days after reprogramming induction may include some iPSCs. Therefore, we then compared the reprogramming behavior in cells that passed or did not pass the *Lgr5* + stage. We performed *Lgr5*-lineage tracing experiments using MEFs derived from *Lgr5*-KI mice with 4-OHT treatment. *Lgr5*-KI mice contain alleles of *Lgr5*-EGFP-IRES-

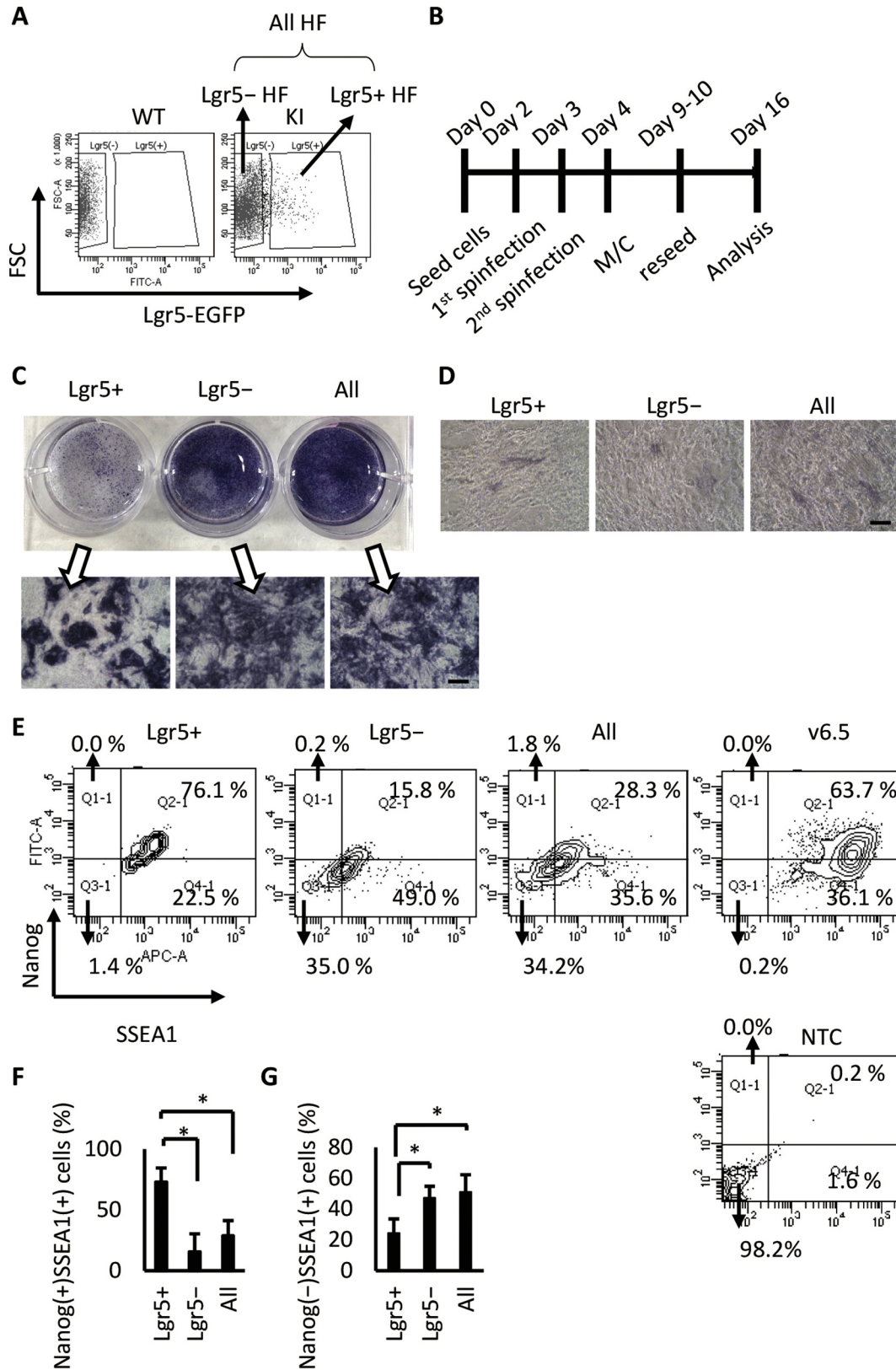


Fig. 1. Lgr5+ hair follicles (HF) convert into Nanog+ cells with high efficiency. (A) Fluorescence activated cell sorting (FACS) plots of Lgr5-EGFP expression in HF. WT = wild type mice, KI = Lgr5-EGFP reporter mice, Lgr5- HF = Lgr5 negative hair follicle, Lgr5+ HF = Lgr5 positive hair follicle, All HF = all hair follicle. (B) The experimental scheme. M/C: medium change. (C) Images of alkaline phosphatase (AP) staining using Lgr5+, Lgr5-, and All HF at 14 days after reprogramming inductions. The bar in the lower image represents 100 μ m. (D) Images of AP staining using Lgr5+, Lgr5-, and All HF without Yamanaka factor transduction. The bar in the image represents 100 μ m. (E) FACS analysis based on the expression of SSEA1 and Nanog using the cell mixture from HF at 14 days after reprogramming induction. v6.5 is a mouse ES cell. NTC is a negative control plot (without primary antibodies). (F) The means \pm SDs of Nanog+ SSEA+ cells (%). n = 4 per group. (G) The means \pm SDs of Nanog- SSEA+ cells (%). n = 4 per group. *p < 0.05.

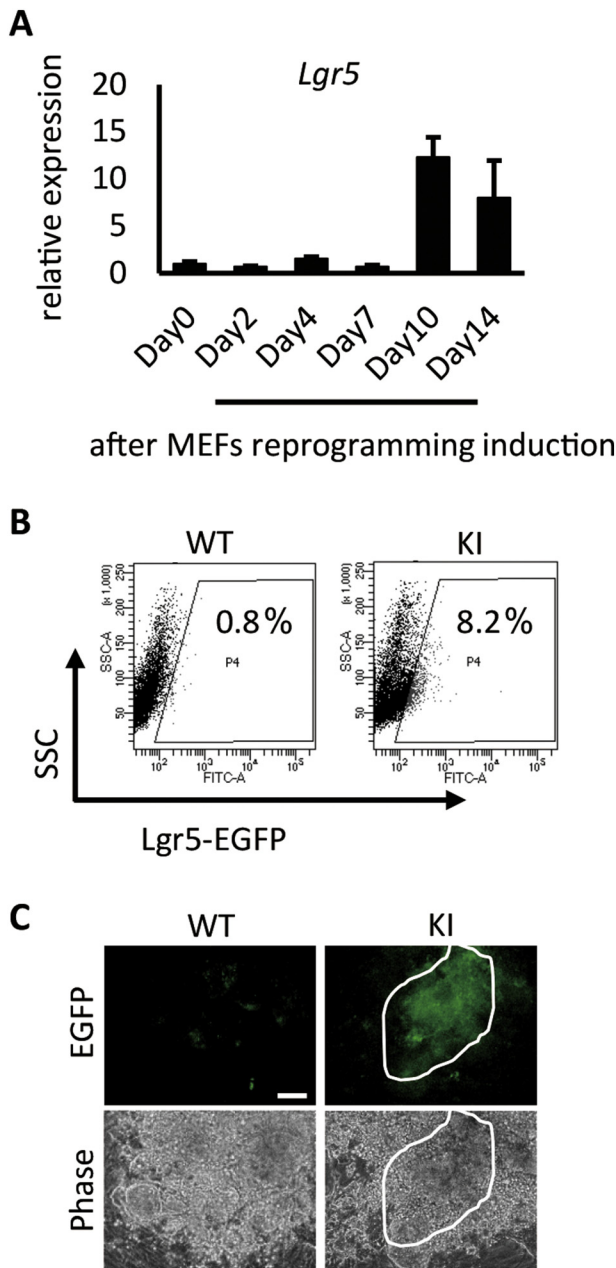


Fig. 2. *Lgr5* expression was transiently upregulated by Yamanaka factor transductions in mouse embryonic fibroblasts (MEFs). (A) The relative mRNA levels of *Lgr5* after reprogramming induction. Values are means ± SDs as normalized to *Actb*. (B) Fluorescence-activated cell sorting plots of *Lgr5*-EGFP expression in MEFs 14 days after reprogramming induction. The numbers in images represent the rate for EGFP positive cells in total cells. (C) Representative images of WT or KI MEFs on day 14 after reprogramming induction. The bar in the image represents 100 μm. A circle of dashed line shows cells positive for EGFP signal. WT = wild type mice, KI = *Lgr5*-EGFP reporter mice.

CreERT2 and *Rosa26-loxp-stop-loxp-LacZ*. Thus, the cells that once expressed *Lgr5* showed *LacZ* activity during 4-OHT treatment. We could distinguish the progeny of M-*Lgr5* + cells by their *LacZ* activity (*LacZ* +). MEFs derived from litters without the *Lgr5* reporter allele were used as a control (*Lgr5*-WT).

The result of *in vitro* X-gal staining at 14 days after reprogramming induction showed that although a few *LacZ* + iPSC-like crowded colonies were observed (0.60 ± 0.89 per well, indicated by a circle of dotted line), almost all *LacZ* + cells formed non-iPSC-like colonies (7.4 ± 2.9 per well, indicated by a circle of dashed line) (Fig. 4A).

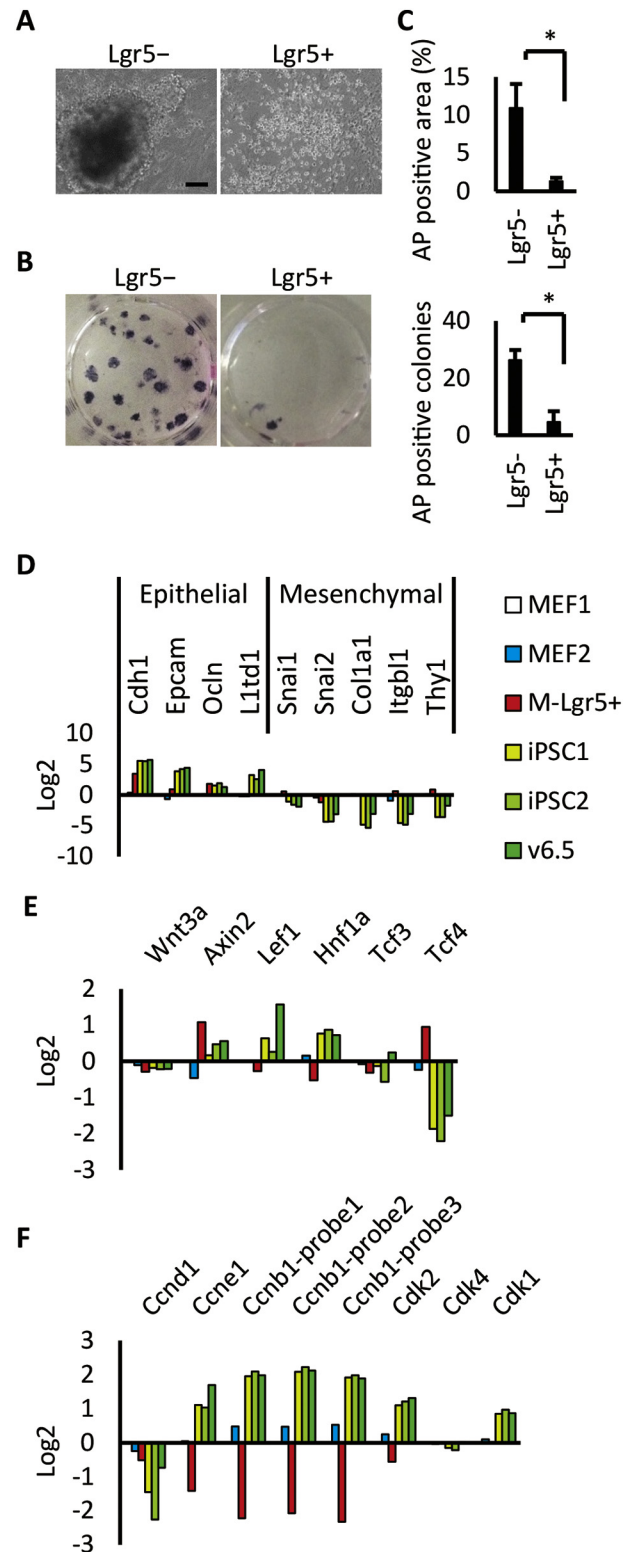


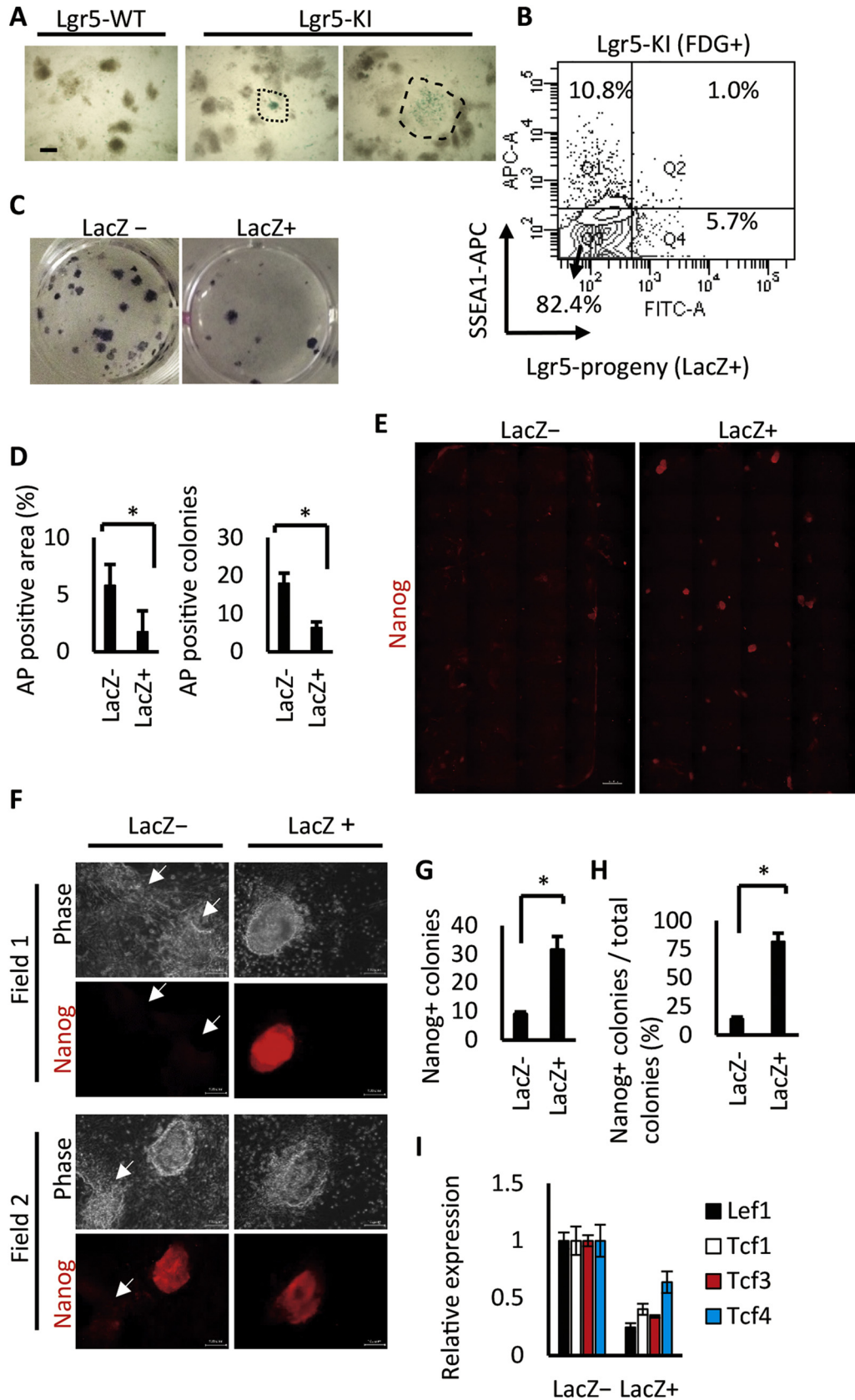
Fig. 3. Evaluation of *Lgr5* + cells derived from mouse embryonic fibroblasts (MEFs). (A) Representative images of M-*Lgr5* - and M-*Lgr5* + cells at 21 days after reprogramming induction. A bar in an image represents 100 μm. (B) Images of alkaline phosphatase (AP) staining in wells of 24-well plates at 21 days after MEFs reprogramming induction. (C) Upper: Means ± SDs of AP + cells area per well (%). Lower: Means ± SDs of the number of AP + colonies per well. n = 3 per group. *p < 0.05 (D-F). The results of microarray analysis. Each bar represents relative value to MEF1.

To assess the fate of the progeny of M-*Lgr5* + cells in detail, we detected the β-galactosidase activity (*LacZ* activity) with fluorescein di β-D-galactopyranoside (FDG) using a flow cytometer 14 days after

MEFs reprogramming induction. The FDG signal could be distinguished from the Lgr5-EGFP signal by its stronger intensity (Fig. S3). To investigate the status of reprogramming of LacZ+ (M-Lgr5+ cells progeny) and LacZ- cells (nonprogeny), SSEA-1 expression was evaluated. SSEA1 was expressed by 14.9% of LacZ+ cells and 11.6% of LacZ-

cells 14 days after reprogramming induction (Fig. 4B). There was a slight difference in the SSEA1+ population between LacZ+ and LacZ- cells.

Next, LacZ+ and LacZ- cells were sorted 14 days after MEFs reprogramming induction using a flow cytometer and seeded onto the feeder cells. Further 7 days later (21 days after reprogramming



induction), LacZ+ cells formed fewer AP+ colonies than LacZ– cells (Fig. 4C and D). We found that the majority of observed colonies in LacZ+ cell wells were well-formed iPSC-like colonies, as observed using Lgr5+ HFs. Thus, we also examined the Nanog expression of the cells derived from LacZ+ and LacZ– cells, respectively. At 14 days after MEFs reprogramming induction, LacZ+ and LacZ– cells were sorted using a flow cytometer and seeded at a density of 5000 cells per well in 4-well chamber slides. Further 7 days later (at 21 days after reprogramming induction), as seen in Fig. 4E–H, the number of Nanog+ colonies was much higher in wells seeded with LacZ+ cells than that in wells seeded with LacZ– cells. Moreover, non-iPSC-like colonies without Nanog expression (indicated by arrows in Fig. 4F) were often observed in wells seeded with LacZ– wells but not in wells seeded with LacZ+ wells (Fig. 4H). In addition, we compared the expression levels of genes involved in stage-specific reprogramming (Ho et al., 2013) between LacZ– and LacZ+ cells 14 days after reprogramming induction. The expression levels of *Tcf3* and *Tcf4*, both of which inhibit the late phase of reprogramming, were lower in LacZ+ cells than those in LacZ– cells (Fig. 4I).

3.4. The progeny of Lgr5+ cells derived from human fibroblasts converted into iPSCs

Finally, we examined whether using human Lgr5+ cells also results in effective reprogramming. We found that the level of *LGR5* mRNA in bulk NHDFs was elevated from 18 days after reprogramming inductions as observed in MEFs (Fig. 5A). It seems to be a transient elevation because human ES cells do not express Lgr5 (McCracken et al., 2014). For further analysis of the Lgr5+ cells derived from NHDFs, we sorted Lgr5+ (hu-Lgr5+) and Lgr5– (hu-Lgr5–) cells 21 days after reprogramming induction using a flow cytometer (Fig. 5B). Sorted hu-Lgr5+ and hu-Lgr5– cells were seeded onto 4-well chamber slides coated with Matrigel and then cultured under the feeder-free condition.

Further 14 days later (at 35 days after reprogramming induction), as seen in Fig. 5C and D, some NANOG+ TRA-1-60+ colonies, both of which are markers for pluripotent stem cells (David and Polo, 2014; Tanabe et al., 2013), were detected only in wells seeded with hu-Lgr5+ cells. To examine whether the colonies derived from hu-Lgr5+ cells have the capacity to form three germ layers, the colonies were picked up and then expanded. Picked colonies can differentiate into three germ layers, confirming that the colonies derived from hu-Lgr5+ cells were iPSCs (Fig. 5E).

4. Discussion

Although Lgr5+ cells in tissue stem cells and progenitors have been relatively well studied (Barker and Clevers, 2010a, 2010b; Barker et al., 2010, 2008, 2007; Carmon et al., 2011, 2012; Chai et al., 2011; da Silva-Diz et al., 2013; de Lau et al., 2014; Fukuma et al., 2013; Gil-Sanchis et al., 2013; Haegerbarth and Clevers, 2009; Huch et al., 2013; Jaks et al., 2008; Schuijers and Clevers, 2012; Shi et al., 2012; Yee et al., 2013), the reprogramming behavior of Lgr5+ cells has not been examined. Here we showed that Lgr5+ cells derived from reprogramming MEFs and NHDFs as well as mouse Lgr5+ HFs promoted the generation of Nanog+ colonies.

Lgr5 expression is often observed at the site of active Wnt signaling (Carmon et al., 2011; Schuijers and Clevers, 2012). Various studies

focusing on the relationship between Wnt signaling and cell reprogramming have been reported. The activation of Wnt signaling results in the promotion of reprogramming (Lluis et al., 2008; Marson et al., 2008). On the other hand, Wnt target genes (*Lef1*, *Tcf1*, *Tcf3*, and *Tcf4*) have stage-specific regulation of reprogramming to iPSCs, where suppression and promotion occurs (Ho et al., 2013). In our study, despite the similar levels of *Wnt3a* and *Axin2* between M-Lgr5+ cells and v6.5 (less than two times), the levels of *Lef1* and *Hnf1a* (*Tcf1*) were lower and the level of *Tcf4* was higher in M-Lgr5+ cells than in pluripotent stem cells (Fig. 3E). Surprisingly, this balance is optimized for promoting the early rather than the late phase of reprogramming (Ho et al., 2013). Recently, it has been reported that the reprogramming process consists of sequential events; the early phase is the initiation of reprogramming, and the late phase is the maturation of reprogramming (Buganim et al., 2013; David and Polo, 2014). Cells that transiently express Lgr5+ seemed to be still under the initiation of reprogramming. In addition, M-Lgr5+ cells showed the low expression levels of genes involved in cell cycles (Fig. 3F). However, as shown in Fig. 3A right panel, M-Lgr5+ cells certainly proliferate, although they do not form crowded colonies. Thus, their reprogramming speed may be very slow. Comparing the progeny of M-Lgr5+ cells with nonprogeny, we found that the progeny represented the status of late reprogramming phase (*Tcf3* and *Tcf4*; low) to a higher degree (Ho et al., 2013) (Fig. 4I), which is consistent with the result of Nanog expression. Overall, it appears that the MEFs passing through the Lgr5+ stage are slowly, which results in less AP+ cells, but carefully reprogrammed, which results in more Nanog+ cells, compared with nonprogeny. The reason for the slight difference in the ratio of SSEA1+ cells between them is probably that SSEA1 is an intermediate marker of initiation–maturation phase (Brambrink et al., 2008). Moreover, there is a possibility that failed reprogramming in an early stage (AP+ but Nanog–) is unlikely to occur in the Lgr5+ cells progeny. To investigate whether Lgr5 itself has an effect on reprogramming, we additionally performed experiments of Lgr5 overexpression and knockdown during MEFs reprogramming. Lgr5 overexpression from the beginning of reprogramming induction had a negative effect not only on the result of AP staining but also on Nanog expression (Fig. S4A–C). In contrast, Lgr5 overexpression and knockdown during the route to reprogramming had no effect on reprogramming status (Fig. S4A, B, E and F). It seems that the negative effect of Lgr5 overexpression from the beginning of the reprogramming reflects the mere overexpression effect of the protein, rather than the function of Lgr5 itself.

Early studies demonstrated that primitive cells are readily converted into iPSCs (Kim et al., 2008; Kleger et al., 2012; Tsai et al., 2011). Thus, we expected that Lgr5+ cells would be susceptible to reprogramming. Unexpectedly, Lgr5+ cells from HFs and MEFs showed more resistance to early reprogramming rather than sensitivity, as demonstrated by the result of AP staining (Figs. 1C and 4C). However, by focusing on Nanog expression, their progeny showed more Nanog+ cells than nonprogeny. These findings suggest that the Lgr5+ stage is a barrier to protect unsuccessful reprogramming. Indeed, recent studies reported that transiently induced factors by reprogramming and not stably induced factors could function as an indicator of the successful cell reprogramming process (Koga et al., 2014; Wang et al., 2013). Because of the leakage of FDG, we could not determine the number of Nanog+ cells that express LacZ activity (M-Lgr5+ cell progeny). Instead, SSEA1 expression, which is a surface marker, was examined (Fig. 4B).

Fig. 4. M-Lgr5+ cells progeny did not form Nanog– colonies. (A) Representative images of X-gal staining at 14 days after MEFs reprogramming induction. The bar in the image represents 300 μ m. Circles of dotted line represent M-Lgr5+ progeny-forming crowded colonies. Circles of dashed line represent M-Lgr5+ progeny-not-forming crowded colonies. (B) Fluorescence-activated cell sorting plots of Lgr5-KI MEFs 14 days after reprogramming induction based on the expression of SSEA1 and LacZ activity. (C) Representative images of alkaline phosphatase (AP) staining in wells of 24-well plates using LacZ– and LacZ+ cells at 21 days after MEFs reprogramming induction. (D) Left: The percentage of AP+ areas. Right: The numbers of AP+ colonies. n = 3 per group. Values are means \pm SDs. *p < 0.05. (E) Montage images with 4 \times 9 images of immunostaining for Nanog using LacZ– and LacZ+ cells at 21 days after MEFs reprogramming induction. A bar in an image represents 1 mm. (F) Representative images of colonies observed in (E). Bars in images represent 100 μ m. Arrows indicate non-iPSC like colonies without Nanog expression. (G) The number of Nanog+ colonies in E was evaluated. (H) The ratio of Nanog+ colonies to total number of colonies in (E) was evaluated. n = 4 per group. *p < 0.05. Data are shown as means \pm SDs. (I) The relative mRNA levels of *Lef1*, *Tcf1*, *Tcf3*, and *Tcf4* using LacZ– and LacZ+ cells at 14 days after MEFs reprogramming induction. Values are means \pm SDs as normalized to *Actb*.

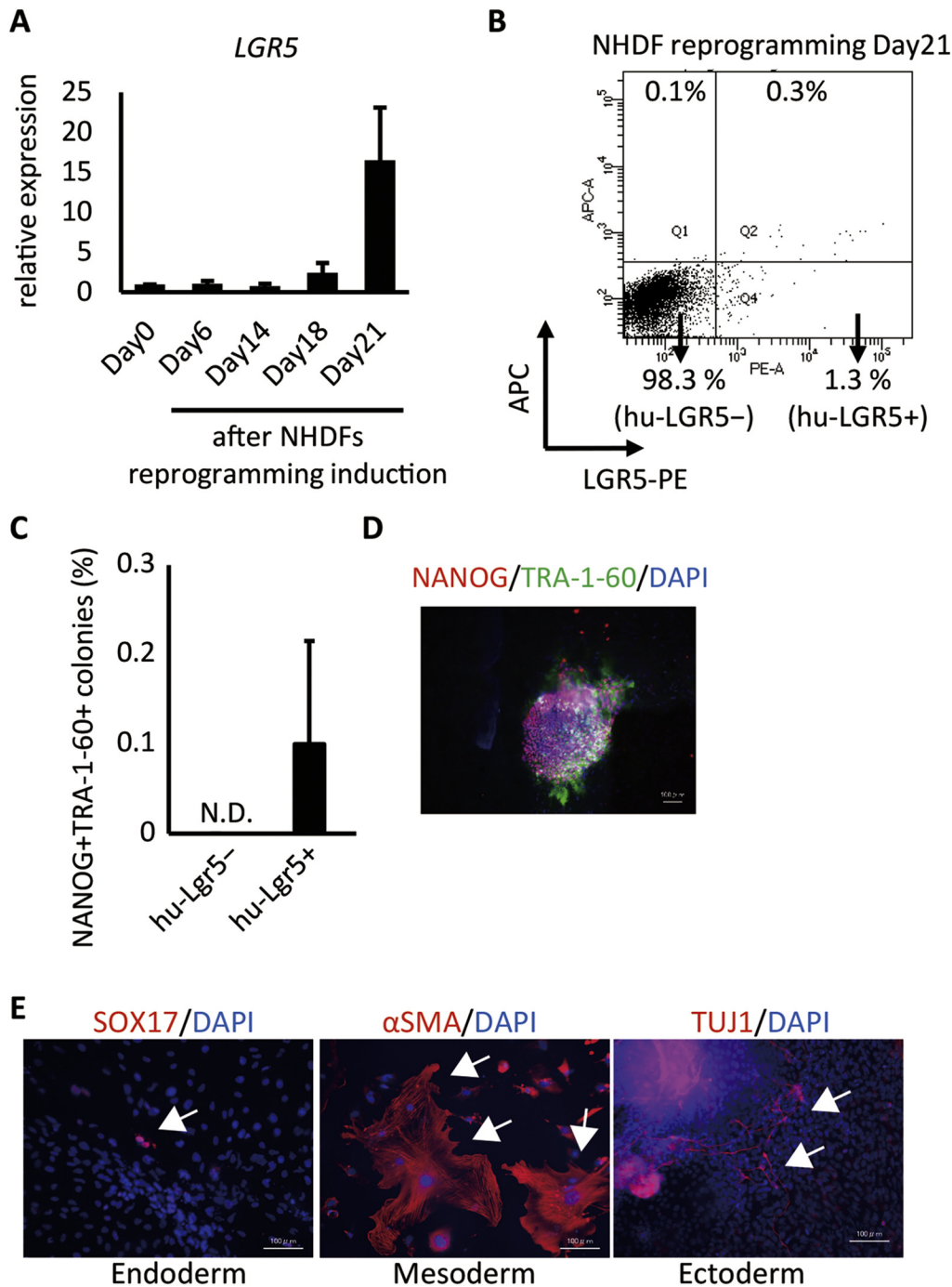


Fig. 5. hu-Lgr5 cells are effectively reprogrammed into iPSCs. (A) The relative mRNA levels of *LGR5* after NHDFs reprogramming induction. Values are means \pm SDs as normalized to *ACTB*. (B) Fluorescence-activated cell sorting plots of Lgr5-PE expression in NHDFs 21 days after reprogramming induction. The numbers in images represent the rate of LGR5-PE positive cells in the cells. (C) The reprogramming efficiency of hu-Lgr5⁻ or hu-Lgr5⁺ cells (% NANOG + TRA-1-60 + colonies of 1500 cells). n = 4 per group. N.D. = not detected. Values are means \pm SDs. (D) A representative image of immunostaining for NANOG and TRA-1-60 using colonies derived from hu-Lgr5⁺ cells at 35 days after NHDFs reprogramming induction. A bar in an image represents 100 μ m. (E) Evaluation for the capacity of the differentiation into three germ layers using iPSC-like colonies derived from hu-Lgr5⁺ cells. This is a representative picture among multi clones. Arrows in images indicate positive cells for each marker. Bars in images represent 100 μ m.

Contrary to the result of the formation of Nanog + colonies, merely 8.4% of SSEA1 + cells showed LacZ activity. This result appears to occur because SSEA1 + cells do not always convert into iPSCs (Brambrink et al., 2008).

5. Conclusion

In conclusion, we found that focusing on Lgr5 positive cells from HFes and MEFs provided hints for facilitating the simple generation of

Nanog + cells without selection, such as using a drug. Lgr5 + HFes will be a promising material for the practical application to humans. Indeed, we found that human Lgr5 + cells can also more effectively convert into successful reprogrammed cells than Lgr5 - cells. In addition, many efforts have been made to increase the reprogramming efficiency (Feng et al., 2009; Tang et al., 2012; Zhu et al., 2014), with most focusing on increasing the number of successfully reprogrammed cells and not on decreasing the number of unsuccessfully reprogrammed cells. Our findings provide a novel strategy for iPSC derivation by inhibiting

unsuccessful reprogramming and contribute to minimizing sorting efforts for obtaining superior iPSCs.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2017.01.012>.

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