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Foxd1-Dependent and Independent Pathways for Reprogramming from Fibroblasts to Induced Pluripotent Stem Cells or Cardiomyocytes

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Abstract

Induced Pluripotent Stem Cells (iPSCs) can differentiate into any cell type. Cardiomyogenesis from iPSCs is useful for clinical application in myocardial regeneration. However, the efficiency and duration of producing iPSCs and iPSC-derived cardiomyocytes must be improved. We previously demonstrated that a surface marker profile of Sca1-CD34- or Foxd1+ during the reprogramming process is a predictor of successful iPSC formation. Here, we examine the correlation of feasibility as iPSC predictors between Sca1-CD34- and Foxd1+ cell populations, and their possibility as predictors for cardiomyocyte transdifferentiation. The fate-tracing analysis revealed that most iPSC colonies were formed from GFP-positive cells in which Foxd1 was transactivated in the middle-to-late phase of the reprogramming process. In addition, GFP expression was observed mainly in the Sca1-CD34- cell population. Thus, Foxd1+ could be an indicator of successful reprogramming to iPSCs mainly derived from Sca1-CD34- cells. As for cardiac transdifferentiation, reprogramming cells were sorted based on the expression pattern of Sca1 and CD34, resulting in a higher incidence of beating cell aggregates derived from the Sca1+CD34+ population, which expresses less Foxd1 promoter-driven GFP and contained very few undifferentiated iPSCs. Moreover, the cardiomyocyte marker α-actinin only partially co-localized with GFP expression in the aggregates derived from Sca1+CD34+ or Sca1-CD34- cells. Therefore, Sca1+CD34+ could be a better cell source for Foxd1-independent cardiomyocyte creation despite the failed reprogramming cell population.

Keywords: Cell reprogramming; Transdifferentiation; Induced pluripotent stem cell; Cardiomyocyte; Foxd1; Sca1; CD34

Abbreviations: CD34: Cluster Of Differentiation 34; EB: Embryoid Body; ESC: Embryonic Stem Cell; EGFP: Enhanced Green Fluorescence Protein; 4F: Four-Factor (Oct4, Sox2, Klf4, And C-Myc); FACS: Fluorescent-Activated Cell Sorting; Foxd1: Forkhead Box D1; iPSC: Induced Pluripotent Stem Cell; MEF: Mouse Embryonic Fibroblast; Sca1: Stem Cell Antigen-1; SSEA1: Stage-Specific Embryonic Antigen-1

Introduction

To establish innovative treatments such as regeneration therapy or personalized medicine,Induced Pluripotent Stem Cells (iPSCs) have extensively been studied throughout the last decade. Somatic cells are reprogrammed to iPSCs by introducing four defined transcription factors (4F: Oct4, Sox2, Klf4, and c-Myc). Reprogrammed iPSCs exhibit unlimited self-renewal and pluripotency in the undifferentiated state like Embryonic Stem Cells (ESCs) [1]. Despite considerable research, the efficiency of iPSC induction remains low and there are potential risks of tumorigenicity and immaturity that limit clinical applications. To overcome these barriers, the underlying mechanisms of iPSC induction must be addressed. Some cell surface markers have been studied in the hope of obtaining iPSC progenitors. Stage-Specific Embryonic Antigen-1 (SSEA1) was reported as a positive marker for iPSC progenitors in the late stages of reprogramming [2-4]. In addition, we found that negative expression of the cell surface markers Stem Cell Antigen-1 (Sca1) and Cluster of Differentiation 34 (CD34) represents an excellent early predictor of iPSC reprogramming. Sca1-CD34- reprogramming cells contain abundant iPSC progenitors in the early phase [5]. Previous work also identified the forkhead box transcription factors, Foxd1, and Foxo1 as reprogramming mediators and indicators, based on a comprehensive analysis of the overrepresented transcription factor binding sites in the promoter sequence of hundreds of genes upregulated after 4F introduction [6]. While Foxo1 was shown to be essential for maintaining pluripotency in ESCs, Foxd1 is known to regulate kidney and retina development [7-10]. Interestingly, Foxd1 deficiency resulted in decreased iPSC production, with Foxo1 knockdown further reducing it [6]. Since the expression patterns of SSEA1, Sca1/CD34, and Foxd1/Foxo1 have been independently investigated as iPSC predictors for successful reprogramming, it remains unknown whether iPSC progenitors defined by these indicators possess similar or different characteristics.

iPSCs are expected to provide cell sources for regenerative medicine. Cardiomyocytes derived from iPSCs have been targeted for clinical applications in the treatment of end-stage heart failure, but the efficiency and duration of iPSC-derived cardiomyocyte production must be improved. iPSC-derived cardiomyocytes are produced through multiple steps, including the collection of somatic cells from the patient, acquiring pluripotency, and differentiation to cardiomyocytes. The entire reprogramming process can take more than a couple of months. One study successfully shortened the process of cardiomyogenesis using the 4F- or 3F- (4F without c-Myc) transducing reprogramming strategy with some modifications [11]. The induction of 4F without leukemia inhibitory factor (LIF), which is used to maintain pluripotency, partially reprogramed somatic cells and

converted the cell fate to other various cell types, including beating cardiomyocyte-like cells [11]. However, it would be of importance to understand how the modified reprogramming process can convert somatic cells to cardiomyocytes. Here, we investigate whether there are shared characteristics between Sca1-CD34- and Foxd1+ reprogramming cells as iPSC predictors and whether other cell populations (e.g., Sca1+CD34+) may preferentially transdifferentiate into cardiomyocyte-like cells.

Materials and Methods

Animals

All animal experiments in this study were approved by the Experimental Animal Committee of Ritsumeikan University. We performed animal care and experimental procedures following the Animal Welfare Committee guidelines of Ritsumeikan University. Mice used in this study were kept in an air- and humidity-conditioned room at 22°C to 24°C in the Animal Facility in Ritsumeikan University. Foxd1-Cre knock-in mice and CAG-loxP-stop-loxP-EGFP transgenic mice were obtained as previously described [6].

These mouse strains were crossed to generate Foxd1-Cre/GAC-loxP-stop-loxP-EGFP double transgenics and the homozygotes were maintained in mouse colonies. E13.5-14.5 embryos were collected via Cesarean section from sacrificed females for the primary culture of Mouse Embryonic Fibroblasts (MEFs).

Cell culture and iPSCs induction

MEFs used as somatic cells for this study were prepared from mouse embryos at E13.5 or E14.5. We cultured MEFs in a medium containing Dulbecco's Modified Eagle Medium (Nacalai Tesque, Japan), 10% fetal bovine serum (Nichirei Bioscience, Japan), 2 mM L-glutamine (GlutaMAX, GIBCO), and 1% (10,000 U/L and 10 mM) penicillin-streptomycin (Wako, Japan). We performed iPSC induction as previously described [5,12]. Briefly, HEK293T packaging cells were transfected with pMXs-mOct4, pMXs-mSox2, pMXs-mKlf4, pMXs-mc-Myc, and pMXs-null, and incubated for 48 h. The virus-containing supernatant of the medium was used for infection. The first day after MEFs were incubated in the virus-containing solution was designated day 0. Two days after infection, cells were maintained in a LIF-supplemented medium to reprogram into iPSCs as previously described [12]. Cell culture was performed at 37°C and 5% CO_2 in a humidified incubator.

Immunocytochemistry

To examine reprogramming efficiency, we performed immunolabeling with antibodies against Nanog in cultured cells on days 11-15 after the introduction of 4F using the VectaStain ABC kit and ImmPACT DAB substrate (Vector Laboratories), as previously described [5,12]. Briefly, the cells were fixed in 10% neutral phosphate-buffered formalin, permeabilized with 0.5% Triton X-100, and blocked with 5% BSA. After overnight reaction with the primary antibodies against Nanog (Calbiochem), the cells were washed and incubated with the biotin-conjugated anti-rabbit IgG (Vector Laboratories) for 45 min and reacted with streptavidin HRP complex for 30 min. Nanog-positive colonies were visualized in DAB solution, washed in distilled water and the number of colonies was counted under a microscope.

Immunofluorescent staining

After cell culture for transdifferentiation, embryoid body (EB)-like cell aggregates were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100 in PBS. After washing in PBS and blocking in 3% normal goat serum-containing PBS, cells were incubated with anti-cardiac α-actinin primary antibodies (Abcam, 1:500 dilution) at 4°C overnight, anti-mouse IgG-AlexaFluor594 secondary antibodies (Thermo Fisher Scientific, 1:500 dilution) at room temperature for 1 h, and 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, 1:3000 dilution) as a nuclear counterstain at room temperature for 3 min. After washing and mounting a coverslip, images were acquired using the BZ-X710 fluorescence microscope with GFP and TRITC filters (Keyence).

Fluorescent-activated cell sorting (FACS)

Cells infected with 4F were sorted using FACS (FACSAria III, BD Biosciences) 5 days after infection using APC-conjugated anti-Sca1 (1:100 dilution, BioLegend), PE-conjugated anti-CD34 (1:100 dilution, BioLegend), and PE- or APC-conjugated anti-SSEA1 (1:5000 dilution, BioLegend) antibodies. After trypsinization, cultured cells were resuspended with antibody-containing PBS with 1% fetal bovine serum (Nichirei Bioscience) and 1 mM disodium edetate at 4°C for 1 h before being washed twice. The cells were resuspended at up to 5.0×10^6 cells/mL in the buffer solution and subjected to FACS. Acquired data were analyzed using the FlowJo software (FlowJo, LLC).

Cardiomyocyte transdifferentiation

Transdifferentiation induction from $Sca1^-CD34^-$ and $Sca1^+CD34^+$ populations sorted from reprogramming cells was performed through the step of EB formation, as previously described with some modifications [12]. Briefly, post-FACS cells were counted and 6,000 cells/well were plated in a 96-well plate as a floating culture. Every other day, 100 μL of the medium was added. After 6 days of suspension cultures, EB-like cell aggregates were formed and transferred into gelatinized 48-well plates (one sphere/well). For attachment cultures sustained for a week, the culture medium was composed of DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum (Nichirei). In subsequent days, the numbers of spontaneously beating EB-like cell aggregates were counted using a microscope. The incidence of beating aggregates was calculated as differentiation efficiency.

Results

To confirm when the Foxd1 gene is activated during reprogramming into iPSCs, we performed fate-mapping experiments using MEFs prepared from double transgenic mice harboring Foxd1-Cre/CAG-loxP-stop-loxP-EGFP cassettes. In this analysis, sustained GFP signal was observed in the cells after Cre expression when the Foxd1 promoter was activated (Figure 1A). Using retroviral vectors, we introduced genes encoding 4F into MEFs to induce reprogramming to iPSCs before tracing GFP expression in the reprogramming cells. Although we did not observe obvious GFP expression until 7 days after infection, we could detect GFP-positive cells 9 days after induction. These GFP-positive cells seemed to be packed and formed into colonies (Figure 1B). Most of the tightly packed colonies that were isolated from the feeder layer were composed of GFP-positive cells. According to earlier studies [5,13], we next examined the number of iPSC colonies based on morphology and Nanog expression without

drug selection. Immunofluorescent labeling using anti-Nanog antibodies revealed that there were Nanog-positive iPSC colonies with or without co-expression of GFP (Figure 2A). We counted 143 colonies containing 62 Nanog-positive iPSC colonies, which were characteristically compact with distinct borders and well-defined edges, and composed of cells with larger nuclei and less cytoplasm than in fibroblasts. In the iPSC colonies, 87% (54/62) of them exhibited GFP expression, as shown in Figure 2B. These results suggest that Foxd1 expression during the reprogramming process contributes to the production of iPSC colonies.

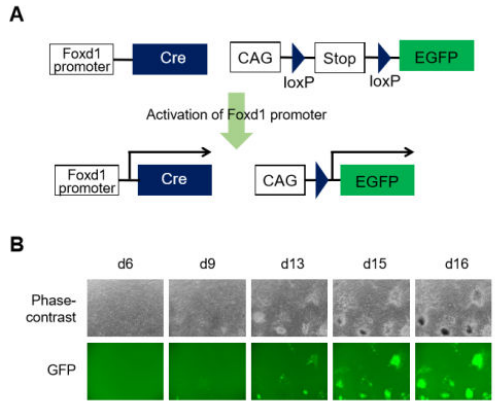

Figure 1: Expression tracing of Foxd1 during the reprogramming process to iPSCs. (A) Illustration of the Cre-loxP system used for GFP-based tracing of Foxd1 gene activation; (B) Microphotographs showing phase-contrast images and GFP expression, reflecting Foxd1-promoter activation during reprogramming to iPSC by 4F 4x objective.

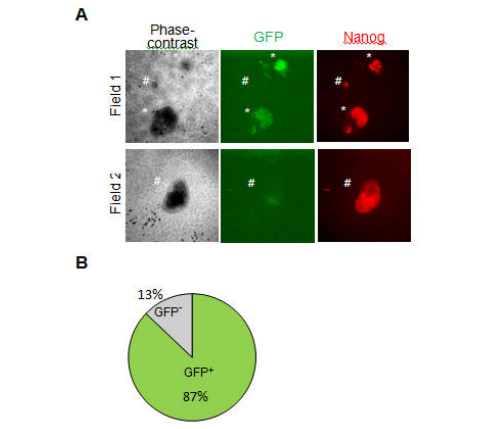

Figure 2: iPSC colony formation from reprogrammed cells with or without Foxd1 expression. (A) Microphotographs of iPSC colonies immunolabeled with anti-Nanog antibodies with (*) or without (#) GFP expression 4x objective; (B) The percentage of GFP positivity in the Nanog-positive colonies.

Our previous work also demonstrates that an expression pattern of cell surface markers Sca1-CD34- can predict a highly reprogrammed cell population at the early phase, but Sca1$^+$CD34$^+$ cells less likely to become iPSCs [5]. Thus, we next investigated whether there are shared properties between Foxd1$^+$ and Sca1$^-$CD34$^-$ cells at each stage of reprogramming. Indicator MEFs harboring Foxd1-Cre/CAG-loxP-stop-loxP-EGFP cassettes were retrovirally infected with 4F and sorted using FACS at each reprogramming time-point. A similar expression pattern of Sca1 and CD34 was observed 5 days after infection as previously reported (Figure 3A) [5]. We continued FACS analysis in the reprogramming cells 6, 9, 13, and 15 days after 4F-transduction. The results showed that a few GFP-positive cells began to appear on day 9, and the increasing rate of the GFP-positive cell population was clearly detected at later time-points (Figure 3B and 3C). These results were in consistency with the results of the fate-mapping experiment (Figure 1B). In terms of correlation with Sca1 and CD34 expression levels, the majority of the GFP$^+$ cell population was observed in both CD34$^-$ and Sca1$^-$ cell populations (Figure 3B and 3C, surrounded by red rectangles). Approximately 70% and 80% of GFP$^+$ cells were negative for Sca1 and CD34 in the late phase of reprogramming on days 13 and 15, respectively (Figure 3B and 3C). These results suggest that Foxd1$^+$ cells might overlap with the Sca1$^-$CD34$^-$ population and be a predictor of successful reprogramming to iPSCs.

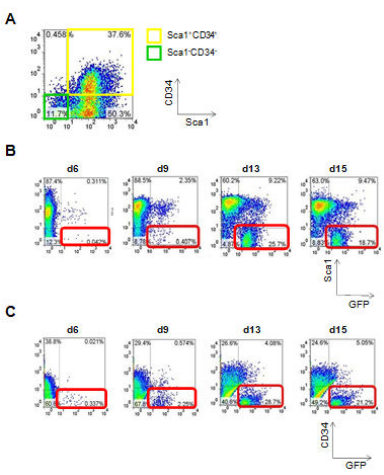

Figure 3: Correlation of the expression level between Sca1 or CD34 and Foxd1 in reprogramming cells. (A) Representative FACS plot in cells based on Sca1 and CD34 expressions; (B, C) Representative FACS plots in cells based on CD34 (B) or Sca1 (C) and GFP expressions.

Since cell conversion to cardiomyocytes can be achieved by modifying the reprogramming process [11], it is interesting to examine whether the reprogramming indicator markers, Foxd1, Sca1, and CD34 are involved in cardiogenesis. Indicator MEFs were infected with retroviruses encoding 4F and sorted using FACS into Sca1$^-$CD34$^-$ or Sca1$^+$CD34$^+$ cell populations five days after infection. The sorted cells were re-plated and cultured in a serum-free medium under low attachment conditions to form EB-like cell aggregates expected to contain cardiac progenitor cells or immature cardiomyocytes. Those cells proceeded to the attachment culture condition for the differentiation step with a serum-containing medium (Figure 4A). Using fluorescence microscopy, we observed that Sca1-CD34--derived cell aggregates showed higher GFP expression than Sca1$^+$CD34$^+$-derived cell aggregates 13 days after 4F infection (Figure 4B). FACS

analysis also showed higher incidence of GFP-positive cells in aggregates derived from Sca1$^-$CD34$^-$ than from Sca1$^+$CD34$^+$ populations (61.0% from Sca1$^-$CD34$^-$ vs 15.3% from Sca1$^+$CD34$^+$, Figure 4C).

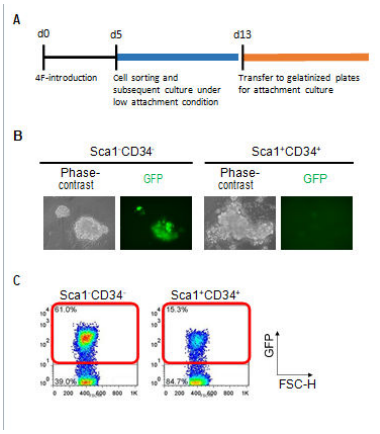

Figure 4: Incidence of GFP-positive cells in DN and DP populations. (A) Microphotographs of phase-contrast and GFP observation of EB-like cell aggregates from Sca1$^-$CD34$^-$ and Sca1$^+$CD34$^+$ populations 20 x objective; (B) Representative FACS plots of cells dissociated from Sca1$^-$CD34$^-$- or Sca1$^+$CD34$^+$-derived cell aggregates. FSC$^-$H: forward scatter-height, indicating cell size.

We monitored the incidence of spontaneously beating cardiomyocytes produced from Sca1-CD34$^-$ and Sca1$^+$CD34$^+$ populations. Although both Sca1-CD34$^-$- and Sca1$^+$CD34$^+$-derived cardiomyocytes started beating on day 23, there was a significantly higher incidence of beating cell aggregates in Sca1$^+$CD34$^+$-derived cells (70%) compared with in Sca1-CD34--derived cells (30%) on day 30 (Figure 5A). Moreover, immunolabeling with cardiac α-actinin, a cardiomyocyte marker, revealed that there was no clear co-localization of α-actinin- and GFP-positive regions, although GFP is co-expressed in fewer regions of α-actinin-positive cardiomyocytes (Figure 5B). These results suggest that the cardiac differentiation observed in this study is Foxd1-independent.

One of the problems associated with iPSC-based cell therapy is contamination of the undifferentiated cells in the cell graft, which can cause a teratoma after implantation. The GFP-positive expression in Sca1$^-$CD34$^-$-derived cell aggregates prompted us to question whether more undifferentiated cells are contained in Sca1$^-$CD34$^-$-derived cell aggregates. To evaluate the incidence of undifferentiated cells detectable in cell aggregates derived from Sca1$^-$CD34$^-$ and Sca1$^+$CD34$^+$ populations, we performed FACS to assess the number of cells expressing SSEA1, a common cell surface marker of undifferentiated pluripotent stem cells. As shown in Figure 6A, 7.2% of Sca1$^-$CD34$^-$-derived cells were SSEA1-positive, but a much smaller percentage (0.8%) of Sca1$^+$CD34$^+$-derived cells were SSEA1-positive (Figure 6A). We further examined the potential contamination of the undifferentiated iPSCs from Sca1$^-$CD34$^-$- and Sca1$^+$CD34$^+$-derived cell aggregates. The aggregates were dissociated into single cells and re-plated on a feeder layer under a suitable condition for undifferentiated pluripotent stem cells. After culturing to maintain the pluripotency in the LIF-containing medium, the cells were fixed and immunostained with anti-Nanog antibodies using the avidin-biotin-peroxidase complex method. The number of iPSC colonies were significantly increased in Sca1$^-$CD34$^-$- than in Sca1$^+$CD34$^+$-derived cell aggregates (Figure 6B). Taken together, these results demonstrate that EB-like cell aggregates from Sca1-CD34$^-$ contain more undifferentiated cells even after the induction of cell conversion to cardiomyocytes. Furthermore, cardiomyocytes produced from Sca1$^+$CD34$^+$-derived cell aggregates may be safer to use in cardiac regeneration therapy.

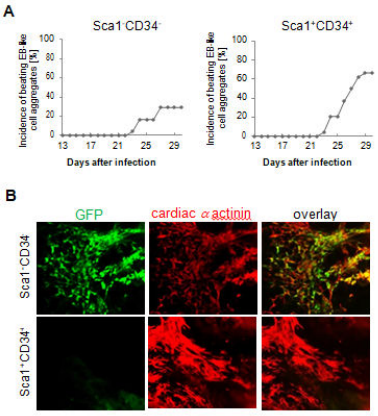

Figure 5: The efficiency of cardiac differentiation in cell aggregates derived from Sca1$^-$CD34$^-$ and Sca1$^+$CD34$^+$ populations. (A) Incidence of beating cells after transgene of 4F; (B) Co$^-$localization of GFP and cardiac α-actinin in beating cells derived from Sca1$^-$CD34$^-$ and Sca1$^+$CD34$^+$ populations 20x objective.

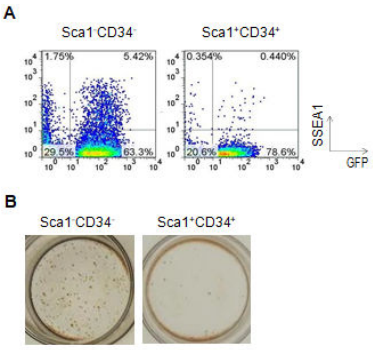

Figure 6: Incidence of undifferentiated cells in cell aggregates from Sca1-CD34- and Sca1$^+$CD34$^+$ populations. (A) Representative FACS plots showing SSEA1 expression in dissociated cells from Sca1$^-$CD34$^-$- or Sca1$^+$CD34$^+$-derived cell aggregates; (B) Photographs of Nanog-positive iPSC colonies produced from the undifferentiated cells contaminating in cell aggregates derived from Sca1$^-$CD34$^-$- or Sca1$^+$CD34$^+$ population.

Discussion and Conclusion

In this study, we performed a Cre-loxP-based fate tracing analysis using Foxd1 promoter-driven Cre expression during reprogramming to either iPSCs or cardiomyocytes. GFP was expressed after Cre-based

recombination in the middle-to-late phase of the reprogramming process to iPSCs. While most Nanog-positive iPSCs were GFP-positive as previously reported, fewer reprogrammed cardiomyocytes were GFP-positive [6]. These results suggest that Foxd1 expression more specifically indicates the precursors to iPSCs than cardiomyocytes. Notably, GFP-positive cells mainly appeared from the $Sca1^{-}CD34^{-}$ population, which are efficiently reprogrammed to iPSCs. Based on our recent study, the $Sca1^{-}CD34^{-}$ population around day 5 was certainly an early predictor of iPSCs [5]. Thus, the results of the present study suggest that iPSC progenitors may be predictable using not only $Sca1^{-}CD34^{-}$ in the early-to-middle phase, but also Foxd1 expression in the middle-to-late phase of reprogramming. Future work investigating whether the $Sca1^{-}CD34^{-}$ population in the middle-to-late phase of reprogramming is iPSC precursor will provide insights on novel roles of Sca1 and CD34 in reprogramming to iPSCs.

Although the $Sca1^{-}CD34^{-}$ population showed successful reprogramming to iPSCs, it remains unknown whether it is also a significant predictor of cardiomyocyte transdifferentiation. Interestingly, cardiac conversion from reprogramming cells sorted based on the expression pattern of Sca1 and CD34 resulted in a higher incidence of beating cardiomyocytes derived from the $Sca1^{+}CD34^{+}$ population than the $Sca1^{-}CD34^{-}$ population. In addition, $Sca1^{+}CD34^{+}$ cells expressed less Foxd1 promoter-driven GFP, and the cardiomyocyte marker α-actinin did not co-localize with GFP expression in cell aggregates either derived from $Sca1^{+}CD34^{+}$ or $Sca1^{-}CD34^{-}$ cells. Moreover, cell aggregates derived from $Sca1^{+}CD34^{+}$ cells exhibit less contamination of undifferentiated cells expressing Nanog compared to $Sca1^{-}CD34^{-}$ cells, and the majority of $Sca1^{-}CD34^{-}$ cells express Foxd1 promoter-driven GFP. Therefore, despite a failed reprogramming to iPSCs, the $Sca1^{+}CD34^{+}$ cell population might be a better cell source for myocardial cell conversion through a Foxd1-independent pathway.

A number of laboratories have been working on the production of cardiomyocytes from other cell types. One prestigious method for direct induction (transdifferentiation) from fibroblasts to cardiomyocytes using gene introduction of the cardiac-specific transcription factors Gata4, Mef2c, and Tbx5 with or without Hand2 has been developed and further improvements have also been reported so far [14-18]. Although this direct reprogramming enables us to generate induced-cardiomyocytes more rapidly and safely due to no use of undifferentiated cells, the reprogramming efficiency by this approach is still limited as induced cardiomyocytes possess the low proliferative capacity. In this study, we focused on more efficient strategies to produce cardiomyocyte from 4F-introduced cells. Various cell culture conditions or protocols for efficient myocardial cell differentiation have been reported. Cardiogenic stimuli-containing media, such as those containing a low concentration of FBS, N2/B27 supplementation, chemical compounds, bFGF, and EGF, significantly improved the efficiency of cardiac differentiation by modulating TGF-β, bone morphogenetic protein, Wnt or Notch signaling [19-22]. According to the previous study, 4F-induced cardiac reprogramming is mediated by cardiac progenitor-like cells expressing Mesp1 or Gata4, which are early mesodermal marker genes [11]. Although further studies are needed to explain how cardiomyocytes are induced from $Sca1^{+}CD34^{+}$ cells, considering the common pathway of cardiac differentiation and transdifferentiation, the combination of these strategies with $Sca1^{+}CD34^{+}$-cell sorting could significantly improve efficient cardiomyogenesis.

In conclusion, we revealed that Foxd1-expressed reprogramming cells share the characteristics of Sca1-CD34- iPSC progenitors, suggesting that Foxd1 could be a feasible predictor for successful reprogramming to iPSCs. Moreover, $Sca1^{+}CD34^{+}$ population, most of which is Foxd1-negative, likely fail to become iPSCs, but exhibits a higher incidence of cardiomyogenesis and less contamination with undifferentiated cells. For terminal cardiac differentiation, some modifications in the protocol will enable the field to improve the quality and quantity of cardiomyocyte production from 4F-introduced reprogramming cells. These improvements will contribute to cardiac regeneration therapy. Our working hypothesis based on the results of the present study is shown in Figure 7.

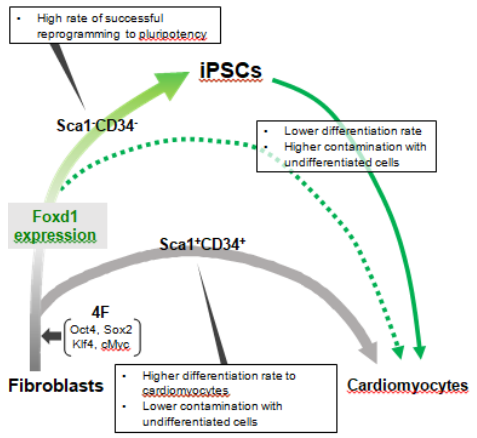

Figure 7: Schematic illustration of our working hypothesis. 4F introduction reprograms fibroblasts to $Foxd1^{+}$ and $Sca1^{-}CD34^{-}$ cell populations, which are successfully reprogrammed to pluripotency. The $Sca1^{+}CD34^{+}$ cell population is also produced, but it fails to be reprogrammed iPSCs. For cardiomyocyte transdifferentiation, $Foxd1^{+}$ and $Sca1^{-}CD34^{-}$ cells show lower efficiency of cardiac conversion and higher contamination with undifferentiated cells, but $Sca1^{+}CD34^{+}$ cells efficiently transdifferentiate to cardiomyocytes with less undifferentiated cell contamination. This suggests that the $Sca1^{+}CD34^{+}$ population is a safer cell source for cardiac regeneration therapy.

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