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Direct reprogramming of mouse fibroblasts into melanocytes

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TITLE:

Direct Reprogramming of Mouse Fibroblasts into Melanocytes

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KEYWORDS:

Direct reprogramming, melanocytes, vitiligo, lentivirus, melanin, transcription factors

SUMMARY:

Here, we describe an optimized direct reprogramming system for melanocytes and a high-efficiency, concentrated virus packaging system that ensures smooth direct reprogramming.

ABSTRACT:

The loss of function of melanocytes leads to vitiligo, which seriously affects the physical and mental health of the affected individuals. Presently, there is no effective long-term treatment for vitiligo. Therefore, it is imperative to develop a convenient and effective treatment for vitiligo. Regenerative medicine technology for direct reprogramming of skin cells into melanocytes seems to be a promising novel treatment of vitiligo. This involves the direct reprogramming of the patient's skin cells into functional melanocytes to help ameliorate the loss of melanocytes in patients with vitiligo. However, this method needs to be first tested on mice. Although direct reprogramming is widely used, there is no clear protocol for direct reprogramming into melanocytes. Moreover, the number of available transcription factors is overwhelming.

Here, a concentrated lentivirus packaging system protocol is presented to produce transcription factors selected for reprogramming skin cells to melanocytes, including Sox10, Mitf, Pax3, Sox2, Sox9, and Snai2. Mouse embryonic fibroblasts (MEFs) were infected with the concentrated lentivirus for all these transcription factors for the direct reprogramming of the MEFs into induced melanocytes (iMels) *in vitro*. Furthermore, these transcription factors were screened, and the system was optimized for direct reprogramming to melanocytes. The expression of the characteristic markers of melanin in iMels at the gene or protein level was significantly increased.

These results suggest that direct reprogramming of fibroblasts to melanocytes could be a successful new therapeutic strategy for vitiligo and confirm the mechanism of melanocyte development, which will provide the basis for further direct reprogramming of fibroblasts into melanocytes *in vivo*.

INTRODUCTION:

Vitiligo is a skin disease that seriously affects the physical and mental health of the affected individuals. For various reasons, including metabolic abnormalities, oxidative stress, generation of inflammatory mediators, cell detachment, and autoimmune response, the functional melanocytes are lost, and the secretion of melanin is stopped, leading to the development of vitiligo[1,2]. This condition occurs widely and is particularly problematic on the face. The main treatment is the systemic use of corticosteroids and immunomodulators. Phototherapy can be used for systemic or local diseases, and there are surgical treatments, such as perforated skin transplantation and autologous melanocyte transplantation[3-5]. However, patients who use drug therapy and phototherapy are prone to relapse, and these treatments have poor long-term therapeutic effects. Surgical treatment is traumatic and only moderately effective[2,6]. Therefore, a new and effective therapeutic strategy is needed for vitiligo.

The reprogramming of induced pluripotent stem cells (iPSCs) reverses these cells from their terminal state to a pluripotent state, a process mediated by the transcription factors, Oct4, Sox2, Klf4, and c-Myc[7]. However, due to the possibility of tumorigenicity and the long production time, this technology has been met with skepticism when applied to clinical settings[8]. Direct reprogramming is a technology that makes one type of a terminal cell transform into another type of a terminal cell[9]. This process is achieved by suitable transcription factors. Various cells have already been directly reprogrammed successfully, including cardiomyocytes[10], neurons[11], and cochlear hair cells[12]. Some researchers have even reprogrammed skin tissue directly *in situ*, which can be used for wound repair[13]. The advantages of direct reprogramming include reduced wait times and costs, lower risk of cancer, fewer ethical problems, and a better understanding of the mechanism underlying cell fate determination[9].

Although the direct reprogramming method is widely used, there is currently no definite method for the direct reprogramming of skin cells into melanocytes, especially because of the numerous transcription factors to be considered[14,15]. The transcription factors, Mitf, Sox10, and Pax3, have been used for direct reprogramming of skin cells into melanocytes[14]. In contrast, the combination of MITF, PAX3, SOX2, and SOX9 has also been used for direct reprogramming of skin cells into human melanocytes in another study[15]. In this protocol, despite the use of a different screening method, the same result was obtained with the combination of Mitf, Sox10, and Pax3 for direct reprogramming of skin cells into melanocytes as described previously[14]. Developing a system to generate melanocytes from other skin cells can provide a scheme for transforming other skin cells of vitiligo patients into melanocytes. Hence, it is crucial to construct a simple and efficient method for this direct reprogramming to generate melanocytes successfully.

PROTOCOL:

This work was approved by the Laboratory Animal Management and Use Committee at Jiangsu University (UJS-IACUC-AP--20190305010). The experiments were performed in strict accordance with the standards established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). There were no experiments involving humans, so this work did not need approval from the human research ethics committee. Refer to the **Table of Materials** for details about reagents.

1. Construction of a concentrated lentivirus packaging system for transcription factors

1.1. Production of the concentrated virus (**Figure 1A, B**)

1.1.1. Plate 1.5 × 10^6 HEK-293T cells into a 60 mm dish and culture these cells with normal medium (see **Table 1**) at 37 °C in a humidified incubator with 5% CO_2.

NOTE: If the virus needs to be packaged in batches, a 100 mm cell culture dish can be used (for details, see **Table 2**).

1.1.2. After 24 h, ensure that the HEK-293T cells have reached 80–90% confluence on the day of transduction, and replace the medium with 3.5 mL of DMEM (150 µL of DMEM per 1 cm^2). Leave the cells at 37 °C in a humidified incubator with 5% CO_2 for 2 h.

NOTE: The replacement medium must be serum-free, so that the cells can be "starved" for better plasmid transfection.

1.1.3. Prepare the mixture of plasmids (mix A) containing 3 µg of the target plasmids of Mitf, Sox10, Pax3, Sox2, Sox9, or Snai2; 1 µg of the packaging plasmid PMD2.G, and 2 µg of the packaging plasmid PSPAX2. Make up the volume of mix A to 150 µL with serum-free DMEM. Prepare the mix of the transfection reagent (mix B) by adding 12 µL of the transfection reagent (the volume is twice the total mass of all the plasmids), and make up the volume of mix B to 150 µL with serum-free DMEM.

NOTE: When preparing the mixes, it is important to add liquid slowly to avoid air bubbles.

1.1.4. Combine mix A and mix B after allowing them to stand for 5 min at room temperature. Incubate the mixture at room temperature for 20–30 min to form the transfection complex.

1.1.5. Take the HEK-293T cells out of the incubator, replace the medium with DMEM + 2% FBS, add the mixture from step 1.1.4 dropwise, and mix the liquid gently.

1.1.6. After 8 h, change the medium with 3.5 mL of normal medium. After changing the medium, collect the virus supernatant every 24 h and 48 h.

1.1.7. Mix the virus supernatants collected at two different time points. Centrifuge at 200 × *g* for 5 min at 4 °C. Pass the supernatant through 0.45 µm filters and collect it in a 50 mL sterile conical

tube.

NOTE: The virus collected at 24 h can be stored at 4 °C and mixed with the virus collected at 48 h.

1.1.8. Concentrate the virus supernatant by centrifuging at 6000 × *g* at 4 °C overnight (~16 h). Ensure that the virus pellet is visible at the bottom of the conical tube after centrifuging.

1.1.9. Pour out the supernatant slowly. Dissolve the virus pellet in a volume of normal medium that is 1/100th of the volume of the virus supernatant. Using a P1000 micropipette, pipette up and down gently until a homogeneous mixture is obtained. Divide the concentrated virus into microcentrifuge tubes as needed. Store at −80 °C.

NOTE: The virus is concentrated 100x with this method. The concentrated virus (100x) can be stored for >1 year at −80 °C. Avoid repeated freezing and thawing of the virus.

1.2. Detection of concentrated virus titer (**Figure 1C**)

1.2.1. Plate 1 × 10^5 HEK-293T cells into one well of a 6-well plate. Remember to add one well as a negative control. Culture these cells with normal medium at 37 °C in a humidified incubator with 5% CO_2.

1.2.2. Add 0.1 µL or 0.2 µL of fluorescent concentrated virus (100x) to each well after 24 h, and add 4 ng/µL of the cationic polymeric transfection reagent to each well. About 8–12 h after infection, replace the medium with normal medium.

NOTE: To ensure the accuracy and efficiency of the fluorescence detection, the infection rate must be 10–30%. Adding 0.1 µL or 0.2 µL of the fluorescent concentrated virus can maintain the infection efficiency within this range. The concentrated virus titer can reach 1 × 10^8 transducing units (TU)/mL at least.

1.2.3. Approximately 48 h after infection, wash the dish with 1 mL of sterile phosphate-buffered saline (PBS) to remove dead cells.

1.2.4. Trypsinize these cells using 250 µL of 0.05% trypsin-EDTA per well in a 6-well plate for 1 min at room temperature. Centrifuge at 200 × *g* for 5 min at 4 °C and then remove the supernatant.

1.2.5. Resuspend the cell pellet in 1 mL of PBS, add the suspension to a 5 mL polystyrene round-bottom tube, and detect the infection efficiency of the virus fluorescence (green fluorescent protein, GFP^+) using flow cytometry.

1.2.6. Calculate the concentrated virus titer using the following formula: 10^5 (cell volume) × infection rate (GFP^+%)/added virus volume (0.1 µL or 0.2 µL).

2. Direct reprogramming of fibroblasts to melanocytes (Figure 2A)

2.1. Coat one well of a 6-well cell culture plate with 1 mL of 0.1% gelatin solution at room temperature for 15–30 min. Ensure that the well is completely covered with the 0.1% gelatin solution. Aspirate the 0.1% gelatin solution after coating.

NOTE: Prepare 0.1% gelatin solution (100 mL) as follows: 0.1 g of gelatin powder is dissolved in 100 mL of ultrapure water in an autoclaved glass bottle and then stored at 4 °C for no more than 2 months.

2.2. Plate 5×10^4 MEFs into one well of a 6-well plate coated with 0.1% gelatin (as in step 2.1), and culture these cells with normal medium at 37 °C in a humidified incubator with 5% CO_2 overnight.

2.3. After 24 h, confirm that the MEFs have reached 40–50% confluence. Replace the medium with normal medium.

2.4. On Day 0, take out the concentrated virus from the freezer and melt the virus on ice. Calculate the volume of the virus to be added by using eq. (**1**). Add the concentrated virus for the six transcription factors, Mitf, Pax3, Sox10, Sox9, Sox2, and Snai2 (see **Table of Materials**) to each well according to the calculated volume, and then add 4 µg/mL of the cationic polymeric transfection agent.

Cell number (5×10^4) × 30 (multiplicity of infection, MOI)/virus titer (**1**)

2.5. On Day 1, 8–12 h after infection, remove the medium containing the virus and replace it with fresh normal medium while adding 0.5 µg/mL puromycin to screen stable infected cell lines.

2.6. On Day 2, 48 h after infection, replace the supernatant medium gradually with the reprogramming medium. First, change $1/4^{th}$ of the total medium volume, and add 3 µM CHIR99021.

2.7. From Day 3 to Day 7, depending on the condition of the cells, change the medium by replacing with a higher proportion of reprogramming medium (see **Table 1**) gradually, and switch to complete reprogramming medium within 5 days.

NOTE: During this period, puromycin and CHIR99021 must be used every day. Many dead cells will appear on the first and second day of changing the reprogramming medium. This is normal as the cells gradually adapt to the transformation. Therefore, the medium needs to be changed gradually to ensure the healthy proliferation of the cells.

2.8. To passage the cells, add 500 µL of 0.05% trypsin-EDTA to digest the cells for 3 min at room temperature. When ~60% of the cells have floated up, stop the digestion by adding normal

medium 2x the volume of the digestive enzyme. Collect the cell suspension in a 15 mL sterile conical tube, centrifuge at 200 × *g* for 5 min at 4 °C, remove the supernatant, resuspend the cell pellet with the reprogramming medium, and plate the cells in a 60 mm sterile dish at a density of $3 \times 10^4/cm^2$. Culture these cells at 37 °C in a humidified 5% CO_2 incubator.

NOTE: From Day 8 to Day 21, these cells are sub-cultured every 3–5 days and cultured in 60 mm sterile dishes to expand. They can be cultured to reach at least passage 5.

3. Optimization for direct reprogramming and identification

3.1. Screening for the optimized transcription factors

3.1.1. Repeat steps 2.1–2.7, reducing one of the six transcription factors each time. Infect the MEFs with the virus with combinations of five transcription factors.

3.1.2. Seven days after infection, extract the RNA of these cells[16], and analyze the expression levels of their melanocytic genes using reverse-transcription PCR (RT-PCR)[17] to screen for the transcription factors with the greatest impact on conversion to melanocytes by removing them one by one (**Figure 3A**).

3.1.3. Use the top three transcription factors impacting the conversion to melanocytes to infect the MEFs. Repeat steps 2.1–2.7.

NOTE: Seven days after infection, the melanocytic genes should be detectable in these transformed cells (**Figure 3B**). Primer information for iMels characterization is included in **Table 3**.

3.2. Identification of induced melanocytes (iMels)

3.2.1. Use immunofluorescence staining[18] to verify that the iMels express melanocytic proteins, including TYRP-1 and DCT (**Figure 4A**).

3.2.2. Melanin-specific 3,4-dihydroxyphenylalanine (DOPA) staining (**Figure 4B**)

3.2.2.1. Prepare 4% paraformaldehyde (10 mL) as follows: dissolve 0.4 g of paraformaldehyde powder in 10 mL PBS. Place the solution in an oven at 56 °C for 2 h to promote dissolution.

NOTE: The solution can be kept at 4 °C for no more than one month.

3.2.2.2. Culture the iMels in a 30 mm dish and wash the dish twice with pre-warmed PBS. Add 1 mL of 4% paraformaldehyde to fix the cells for 20 min, and wash the dish 3 times with PBS.

3.2.2.3. Prepare 0.1% DOPA stain solution (10 mL) just before use by dissolving 0.01 g of L-DOPA powder in 10 mL of PBS. Place the solution in a water bath at 37 °C for 30 min; shake it several

times to promote dissolution.

3.2.2.4. Add 1 mL of freshly prepared 0.1% DOPA staining solution. Incubate in an oven at 37 °C for 2–5 h. If there are no brown-black particles, continue to incubate at 37 °C for another 2 h but not for >5 h. Check the samples every 30 min.

3.2.2.5. Wash the dish 3 times with PBS for 1 min each time. Stain the nucleus with 1 mL of hematoxylin staining solution for 2 min.

3.2.2.6. For long-term storage, dehydrate the samples using 95% ethanol for 3 min and then 100% ethanol for 5 min. Seal the dish with xylene and neutral balsam.

3.2.3. Melanin-specific Masson-Fontana staining (**Figure 4B**)

3.2.3.1. Plate iMels in a 30 mm dish and fix the cells with 4% paraformaldehyde for 20 min; wash the dish 3 times with PBS.

3.2.3.2. Add 1 mL of solution A (ammonia silver solution) from the Masson-Fontana staining kit (see **Table of Materials**), place the dish in a dark box, and place the dark box in an oven at 56 °C for 15–40 min.

NOTE: If brown-black particles are not visible after 15 min in the oven, return the samples to the 56 °C oven to continue incubating but not for >40 min. Some water can be added to the dark box to prevent drying up.

3.2.3.3. Aspirate solution A and wash the dish 5–6 times with distilled water, 1–2 min each time.

3.2.3.4. Add 1 mL solution B (hypo solution) from the Masson-Fontana staining kit, and leave the dish at room temperature for 3–5 min.

3.2.3.5. Aspirate solution B and wash the dish with tap water 3 times, 1 min each time.

3.2.3.6. Add 1 mL of solution C (neutral red dye) from the Masson-Fontana staining kit, and leave the dish at room temperature for 3–5 min.

3.2.3.7. Aspirate solution C and wash the dish 3 times with distilled water, 1 min each time.

3.2.3.8. Add 1 mL of 100% ethanol for rapid dehydration, and aspirate the ethanol after 3 min.

NOTE: Stained samples can be stored for a long time after sealing with xylene and neutral balsam.

REPRESENTATIVE RESULTS:

This article includes the protocols of a concentrated lentivirus packaging system to produce

lentivirus of transcription factors for direct reprogramming of fibroblasts to melanocytes and protocols for screening for transcription factors and direct reprogramming of melanocytes from MEFs.

The success of concentrated lentivirus production was evaluated by observing the fluorescence intensity of GFP (**Figure 1A**) or by flow cytometry (**Figure 1B**) after infecting HEK-293T cells for 48 h with unconcentrated lentivirus (1x) and concentrated lentivirus (100x). The titer of the concentrated virus was 10^8 TU/mL or more, which is relatively high (**Figure 1C**).

Direct reprogramming of fibroblasts to melanocytes is achieved through the infection with transcription factor lentivirus and transformation with the optimized reprogramming medium. The scheme for the generation of iMels from MEFs is shown in **Figure 2A**. Cell morphology gradually changes during direct reprogramming. Cell synapses become elongated, and cell nuclei enlarge. However, these cells gradually age after ~Day 20 (~5 passages) (**Figure 2B**).

One transcription factor was removed at a time from the original set of six transcription factors (Mitf, Pax3, Sox10, Sox9, Sox2, and Snai2) to determine the transcription factor with the greatest impact on reprogramming. Removal of Mitf, Pax3, or Sox10 resulted in the silencing of the expression of melanocytic genes *Tyr*, *Tyrp1,* and *Mlana* (**Figure 3A**), indicating that these three transcription factors had the greatest impact on fibroblast conversion to melanocytes. The expression of melanocytic genes induced by direct programming with three transcription factors was higher than that of all six transcription factors (**Figure 3B**).

Finally, the characteristics of the iMels obtained by using this optimized system of direct reprogramming were identified. The expression of melanocytic markers (TYR, TYYP1) was detected using immunofluorescent staining (**Figure 4A**). Melanin-specific staining methods, including DOPA and Masson-Fontana staining, also showed positive results (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Production of concentrated virus and estimation of titer. (**A**) Fluorescence intensity of GFP after infecting HEK-293T cells with unconcentrated lentivirus (1x) and concentrated lentivirus (100x) (**B**) Comparison of the infection rates of unconcentrated lentivirus (1x) and concentrated lentivirus (100x) as detected by flow cytometry. (**C**) Titer estimation of concentrated lentivirus. Scale bars = 250 µm. Abbreviations: GFP = green fluorescent protein; TU = transducing units.

Figure 2: Generation of iMels from MEFs. (**A**) Schematic diagram of iMels generation. (**B**) Changes in cell morphology during conversion from MEFs to iMels in direct reprogramming on day 0, day 3, day 10, and day 20+. Scale bars = 100 µm. Abbreviations: iMels = induced melanocytes; MEFs = mouse embryonic fibroblasts.

Figure 3: Screening for optimized transcription factors. (**A**) qRT-PCR of *Tyr*, *Tyrp1,* and *Mlana* mRNA levels in cells transduced with five transcription factors (one of the original six

transcription factors has been removed). MEF + EV and MEF + 6F are used as negative control and positive control, respectively. The mRNA expression is normalized to *Gapdh* expression. (**B**) qRT-PCR of *Tyr, Tyrp1,* and *Mlana* mRNA levels in cells transduced with the original six transcription factors and with three transcription factors. MEF, MEF + EV, and MMC are used as a blank, negative control, and positive control, respectively. The mRNA levels are normalized to *Gapdh* levels. All values are mean ± SD of three independent experiments. The primer sequences are shown in **Table 3**. Abbreviations: qRT-PCR = quantitative reverse-transcription PCR; MEF = mouse embryonic fibroblasts; MEF+EV = mouse embryonic fibroblasts + empty vector; MMC = mouse melanocyte; 6F = six transcription factors comprising Mitf, Pax3, Sox10, Sox9, Sox2, and Snai2; 3F: three transcription factors comprising Mitf, Pax3, and Sox10.

Figure 4: Functional identification of iMels. (**A**) Immunostaining of melanocytic markers (TYR, TYYP1) in iMels. Scale bar = 50 µm. See the **Table of Materials** for the dilution of antibodies used in this study. (**B**) Masson-Fontana staining and DOPA staining. MEF and the Melan-a cell line are used as negative control and positive control, respectively. Scale bar = 50 µm. Abbreviations: iMels = induced melanocytes; DOPA = 3,4-dihydroxyphenylalanine; MEF = mouse embryonic fibroblast.

Table 1: Components of normal and reprogramming media.

Table 2: Details of the lentivirus packaging system.

Table 3: Primer information.

DISCUSSION:

The quality of the virus is crucial for the success of direct reprogramming to melanocytes in this protocol. The method of packaging and concentrating viruses in this protocol is simple and easy to repeat and does not rely on any other auxiliary concentrated reagent. This protocol can be followed successfully in most laboratories. To ensure the quality of the concentrated virus, the following points need special attention. One is the cell status of HEK-293T. Although HEK-293T cells are immortalized cells, the cells used to make the concentrated virus must be healthy cells within 10 passages (the titer decreases with higher passages).

Another critical point is the proportion of the transfection reagent (in this case, lipofectamine) used. As cells are damaged after adding the transfection reagent, the ratio of the reagent to plasmids must be checked repeatedly to ensure the optimal condition of the cells and the quality of the virus. The 2:1 ratio of transfection reagent to plasmids is most suitable for HEK-293T cells for packaging the virus in this system. In addition, all steps require gentle handling throughout the process. As virus particles can be absorbed after coating, which affects the virus titer, it is not advisable to coat the dish with any matrix when packaging the virus. Due to the possibility of detachment of HEK-293T, the supernatant must be replaced carefully, especially when fresh medium is added after collecting the virus supernatant after 24 h. Some other considerations include cell density, the length of the transfection time, and the serum content in the medium. These are important issues that affect transfection efficiency. The conditions presented in this

protocol are the most suitable, based on results obtained after many repeated experiments.

In the process of direct reprogramming to melanocytes, it is important to consider the state of the original cell MEFs and the density of MEFs used for direct reprogramming. Before starting the direct reprogramming, MEFs must be cultured in non-coated cell culture dishes. Cells in the proliferation phase (40–50% confluence) should be selected for direct reprogramming; the infection efficiency decreases with increasing cell density. The reprogrammed cells need to be plated in gelatin-coated culture dishes.

The length of time needed for the virus to infect cells is also critical; too long an infection time will impair cell survival, whereas too short an infection time will reduce the efficiency. Eight hours were found to be appropriate for the concentrated virus to infect MEFs in this protocol. The last critical point is the correct way of changing the reprogramming medium. MEFs need to adapt to a new medium. The state of the cells must be checked every day, and the medium must be changed carefully according to the cell status. Changing the reprogramming medium too quickly will cause a large number of cells to die.

When cultivating and subculturing iMels, the passage density of the cells is critical. iMels need a relatively high density to maintain growth. Cell synapses should be in contact with each other for the normal proliferation of these cells. If the density is too low, the cells may stop proliferating. Here, the density of $3 \times 10^4/cm^2$ was found to be a suitable passage density for iMels. After 5 passages, the iMels begin aging (**Figure 2B**); the melanin is more mature at this time, and the resultant cells can be used for immunofluorescence, DOPA, or Masson-Fontana staining. Earlier passages of cells can be used for RT-PCR because the expression of genes will change at a relatively early stage.

Although direct reprogramming is widely studied, there is little research on the direct reprogramming of fibroblasts to melanocytes. Different studies have used different transcription factors[14,15], leading to much confusion. This protocol explains how to produce high-quality concentrated viruses and screen several transcription factors to select the most important ones for direct reprogramming to melanocytes. The medium was supplemented with nutritional factors for the direct reprogramming to melanocytes in this protocol (**Table 2**). Finally, functional iMels were identified successfully. The clear and optimized melanocyte direct reprogramming system could provide new treatment strategies for depigmentation diseases such as vitiligo.

However, there are still some limitations to the technology presented in this article. First, it is challenging to estimate the efficiency of this protocol. CRISPR-Cas9 gene editing could be combined with this protocol to knock in melanocytic genes, such as *Tyr* or *Tyrp-1,* into the initial cells to observe the percentage of induced cells during the reprogramming process. In addition, the lentivirus introduction system used in this protocol may carry the risk of gene recombination and insertion mutation[19]. In the future, more efficient and safer introduction methods could be used, such as non-viral recombinant protein expression vectors or mRNA vectors. The experiment in this protocol is still at the *in vitro* stage. The next step is to reprogram the melanocytes directly *in vivo*, causing non-pigmented skin to become pigmented and to use the direct reprogramming

system to design an effective treatment of vitiligo.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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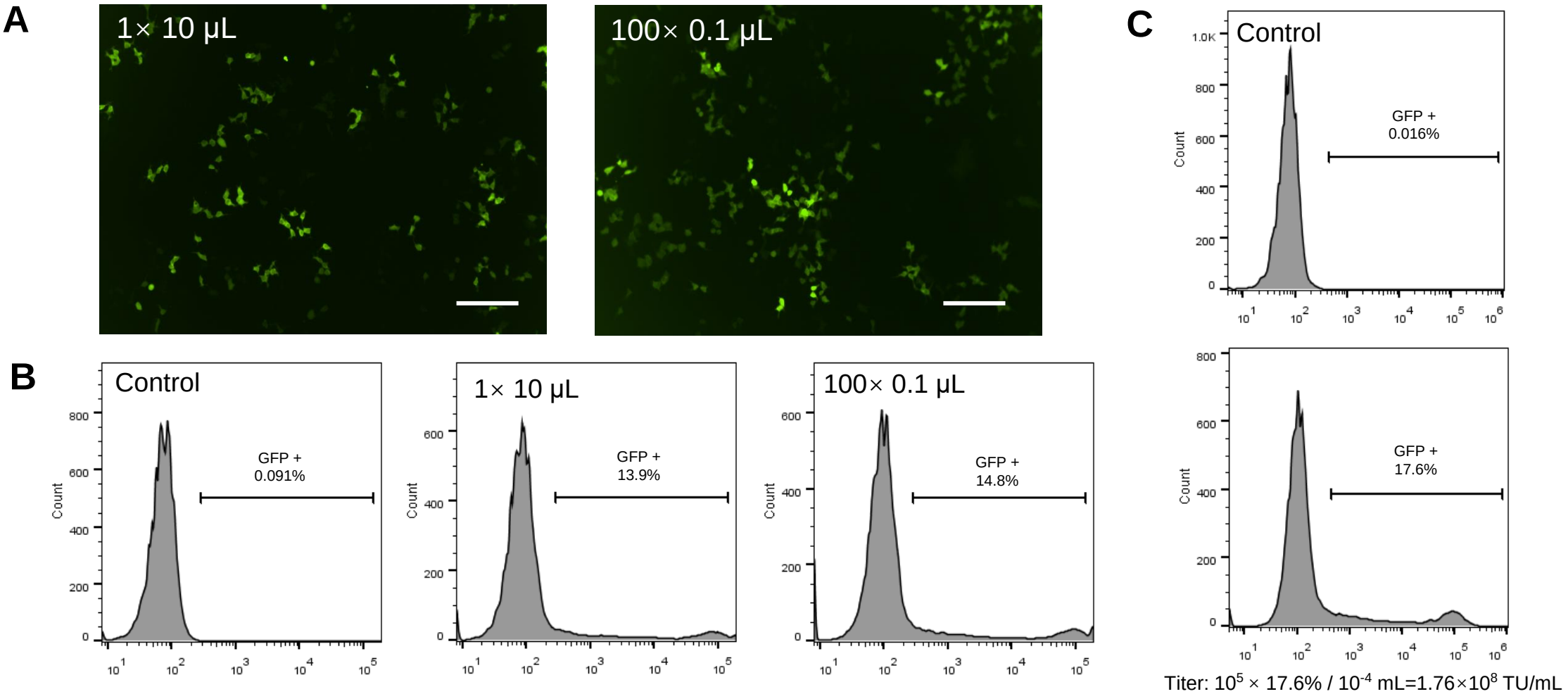
Figure 1



Figure

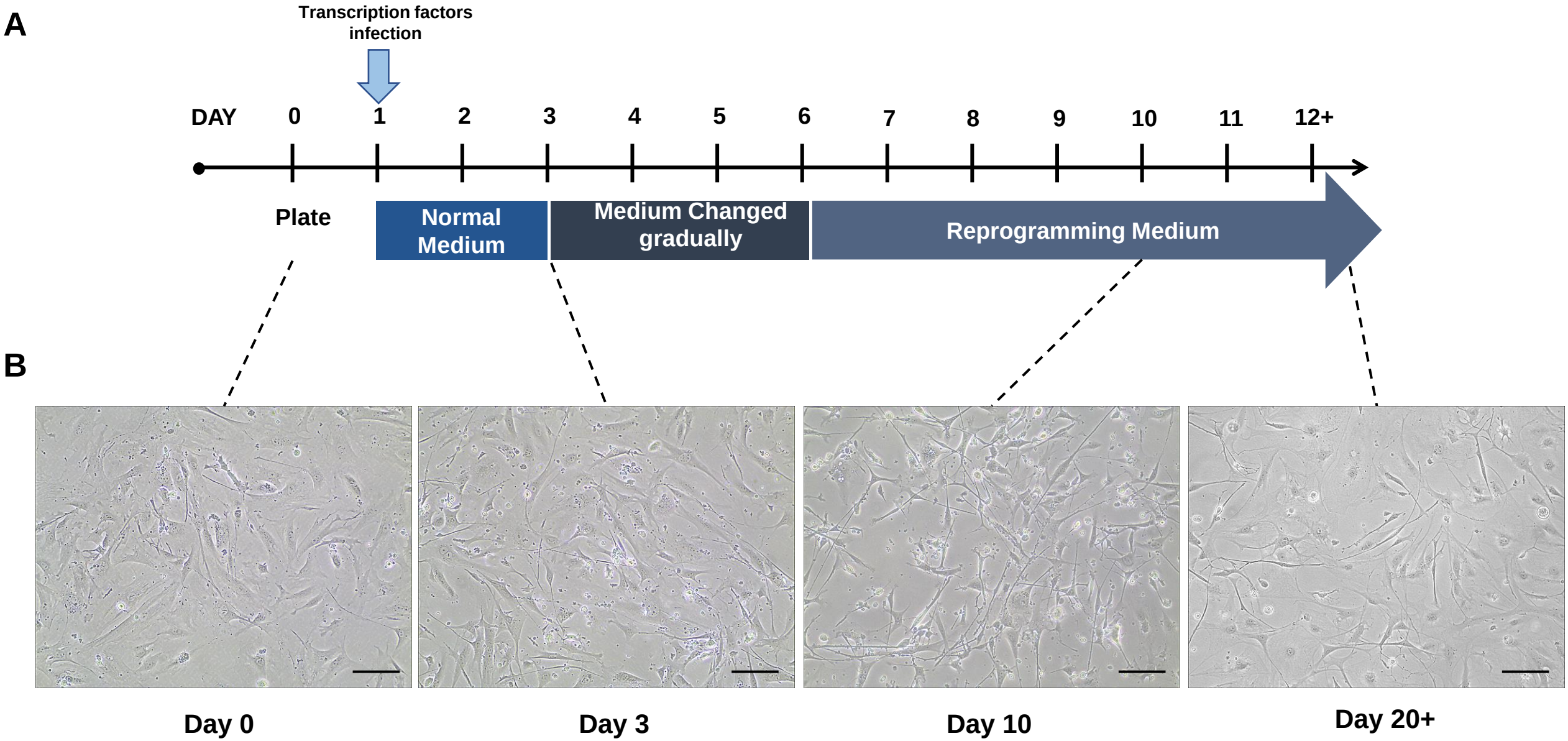


Figure 3

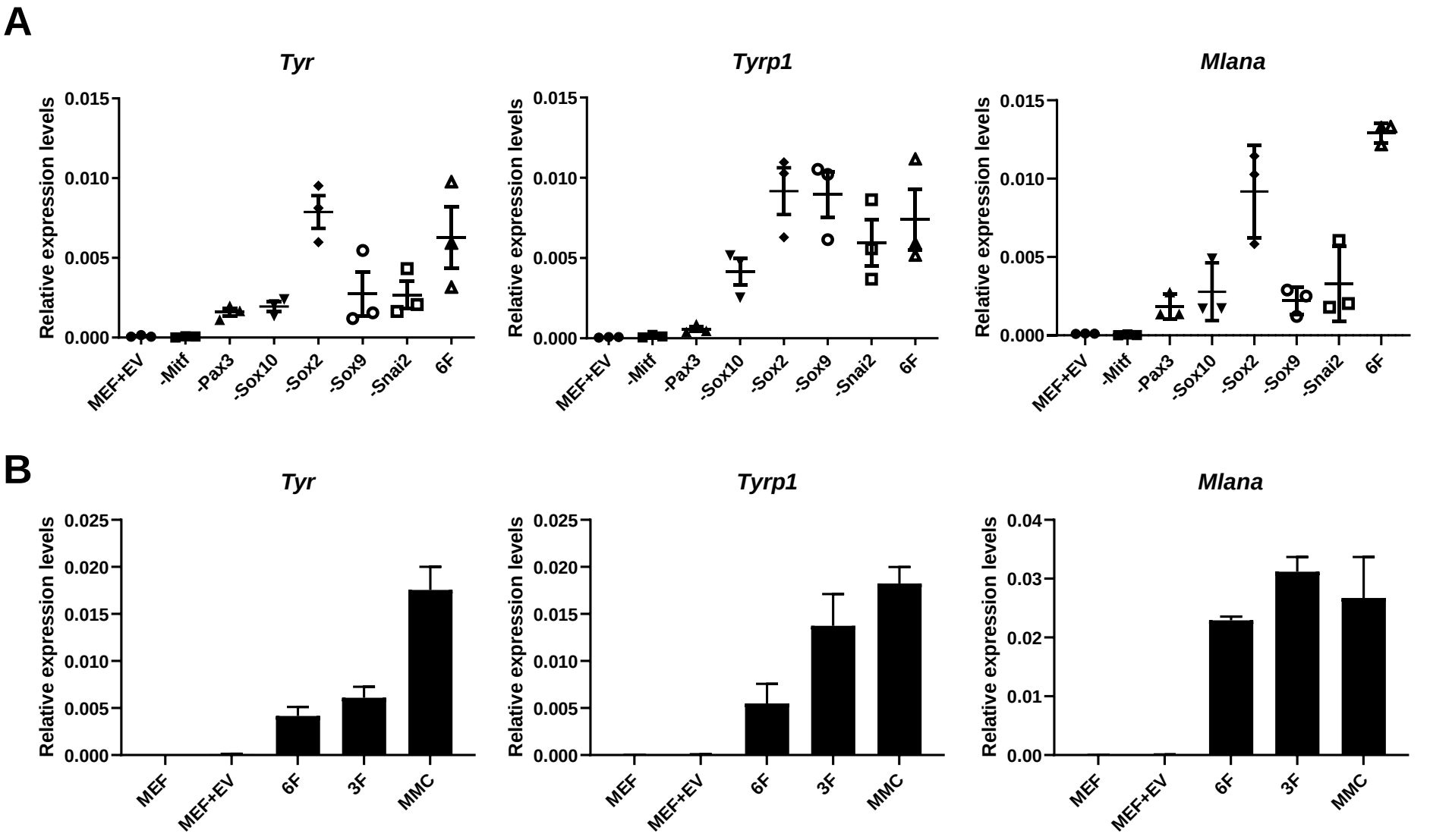


Figure 4

Click here to access/download;Figure;jove Fig 4.pdf

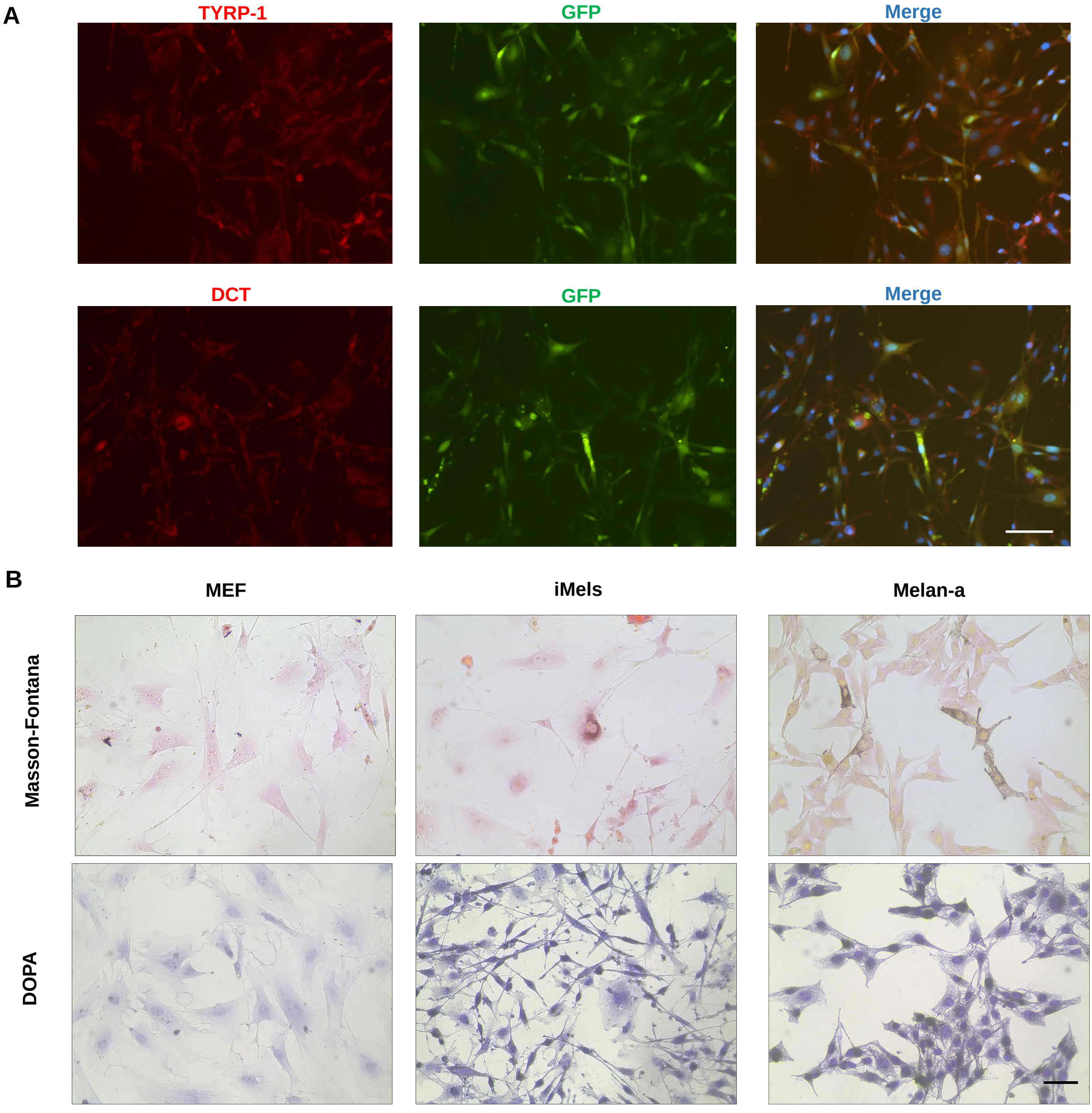


Table 1

	Components	Dose (concentration, volum	Final Concentration
Normal Medium (100 mL)	DMEM/HIGH GLUCOSE	89 mL	
	Heat-inactivated FBS	10 mL	
	Antibiotics(Pen/Strep)	10000 U/mL, 1 mL	100 U/mL
Reprogramming medium (100 mL)	RPMI-1640	88 mL	
	Heat-inactivated FBS	10 mL	
	Recombinant Human SCF	200 µg/mL, 50 µL	100 ng/mL
	Recombinant Human bFGF	4 µg/mL, 250 µL	10 ng/mL
	Recombinant Human insulin	10 mg/mL, 50 µL	5 µg/mL
	EDN3 human	100 µM, 100 µL	0.1 µM
	Cholera Toxin	0.3 mg/mL, 0.56 µL	20 pM
	Phorbol 12-myristate 13-acetate (TPA)	1 mM, 20 µL	200 nM
	Hydrocortisone	100 µg/mL, 500 µL	0.5 µg/mL
	Adenine	40 mg/mL, 60 µL	24 µg/mL

Table 2

Culture Dish	**Seeding Density (cells/dish)**	**Medium (mL)**	**Plasmid System**	
			Target plasmid (μg)	PMD2.G (μg)
35 mm	6×10^5	1.5	1.5	0.5
60 mm	1.5×10^6	3.5	3	1
100 mm	4×10^6	8	8	3

PSPAX2 (μg)	**Transfection reagent (μL)**
1	6
2	12
6	34

Table 3

RT-PCR Primers
Target
Gapdh
Tyr
Tyrp1
Mlana

Primer sets
Forward: CAACGACCCCTTCATTGACC
Reverse: CATTCTCGGCCTTGACTGTG
Forward: GGGCCCAAATTGTACAGAGA
Reverse: ATGGGTGTTGACCCATTGTT
Forward: AAGTTCAATGGCCAGGTCAG
Reverse: TCAGTGAGGAGAGGCTGGTT
Forward: AGACGCTCCTATGTCACTGCT
Reverse: TCAAGGTTCTGTATCCACTTCGT

Dear Dr. Saha:

Thank you very much for your professional supports.
I am the corresponding author Yu-Mei Li of the manuscript “Direct reprogramming of melanocytes with optimization” (MS NO: JoVE62911). Thank you very much for all the comments from the editor and peer reviewers, and I have revised the manuscript based on these comments and responded point by point as following.

Sincerely yours

Yu-Mei Li

Jul. 20, 2021

Response to the editorial comments:
Comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words. The word limit is exceeding in the present Summary.
3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
4. Please ensure that abbreviations are defined at first usage.
5. Please add more details to your protocol steps:
Line 148: Replace “Eppendorf” tube with “microcentrifuge” tube.
Step 1.2.5: Please provide more details regarding the flow cytometer measurement if this step needs to be filmed.
Step 3.1.2: Please provide a citation here regarding RNA extraction.
Step 3.2.1: Please provide a citation here regarding immunofluorescence staining
6. Please include a paragraph on the limitations of the technique in the Discussion section.
7. Please spell out the journal titles in the References.

Responses:
1. According to editorial comment 1, I have proofread the manuscript and modified the spelling and grammar issues;
2. According to editorial comment 2, I have revised the summary to less than 50 words;
3. According to editorial comment 3, I have revised the text to avoid using of personal pronouns as far as possible;
4. According to editorial comment 4, I have checked the text thoroughly to ensure that abbreviations are defined at first usage;
5. According to editorial comment 5

Step 1.1.9: I have replaced “Eppendorf” tube with “microcentrifuge” tube;
Step 1.2.5: This part will not be filmed, mainly demonstrated in the form of the results picture (Figure 1);
Step 3.1.2: I have added the citation regarding RNA extraction;
Step 3.2.1: I have added the citation regarding immunofluorescence staining.

6. According to editorial comment 6, I have added a paragraph on limitations of the technique;
7. According to editorial comment 7, I have spelled out the journal titles in the Reference.

Response to reviewer #1

Comments:

Major Concerns:

1. Since the authors highlight their virus packing method in their manuscript, they need to specify the advantage of their concentrated virus compared with normal virus packing or non-concentrated virus. For example, does concentrated virus help to generate iMels with higher efficiency? The overnight centrifugation is not common in the usual experimental steps, is it necessary to process 16 hours? Is it better than ultracentrifuge?
2. Is there any reason or evidence to support the infection step of using MOI of 30? This is an extremely high MOI compared with others' reports.
3. In Figure 3A, they should include the group of 6F as positive control for readers to understand the gene expression change upon the withdrawal of each transcription factor.
4. In Figure 4, they should include negative control for all staining experiments.

Minor Concerns:

1. The authors should specify that they used three transcription factors to perform the direct reprogramming instead of "as few transcription factors as possible".
2. The authors should find a native speaker to modify and improve their English writing.

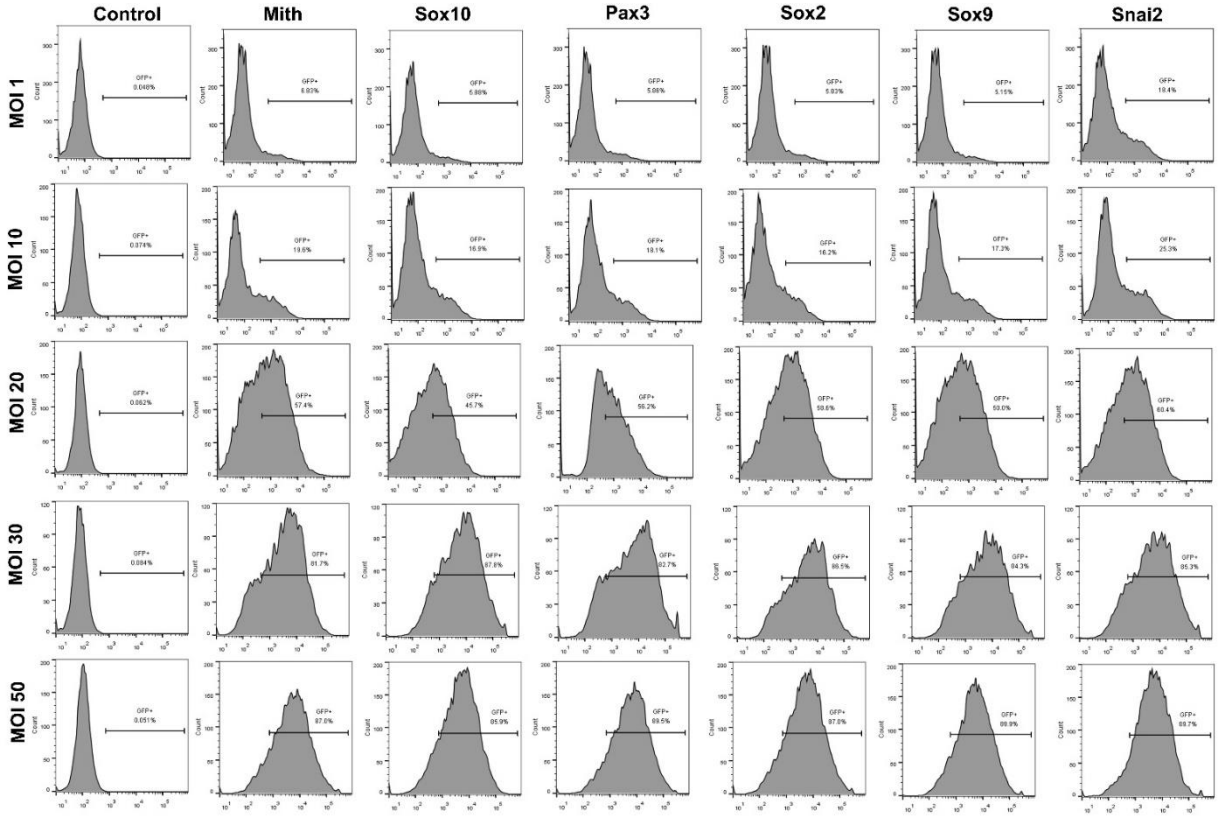
Responses:

Major Concerns:

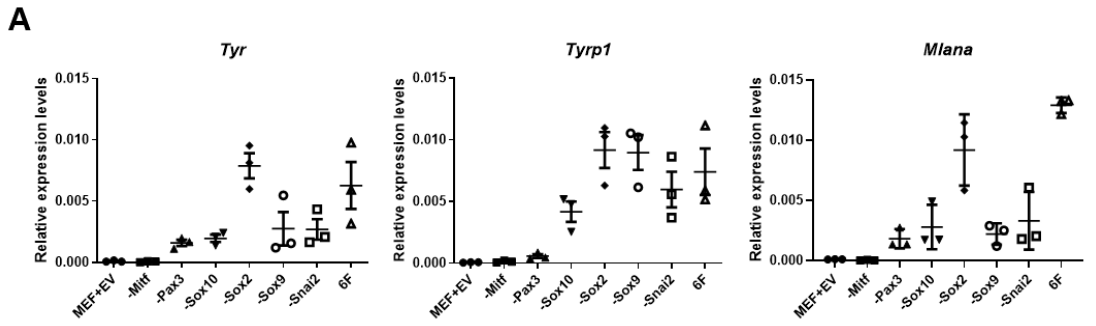
1. According to the major concern 1, the comparison of infection rate between unconcentrated lentivirus (1×) and concentrated lentivirus (100×) has already demonstrated in the Figure 1. Regardless of the results of fluorescence intensity or flow cytometry, the infection efficiency of adding 100 μL unconcentrated lentivirus and adding 1 μL concentrated lentivirus is about the same. Concentrated lentivirus was chosen because adding a small amount of virus fluid would yield more infected cells, since the MOI of MEF infection is relatively high and the amount of virus needed is large, concentrated lentivirus is a better choice. In addition, after our practice of overnight centrifugation, it is an effective way to carry out virus concentration. However, 16 hours is not a very fixed time, and 12-16 hours is acceptable. As for the ultracentrifuge method, we have not tried it,

since our lab protocol and experiences followed from Dr. Yun-Wen Zheng, his lab optimized this practical protocol which originated from Dr. Onodera at NIH (Chiba T, Zheng YW, et al. Gastroenterology 2007;133(3):937-50. PMID: 17673212; Iwama A, Oguro H, et al. Immunity 2004;21:843-51. PMID: 15589172).

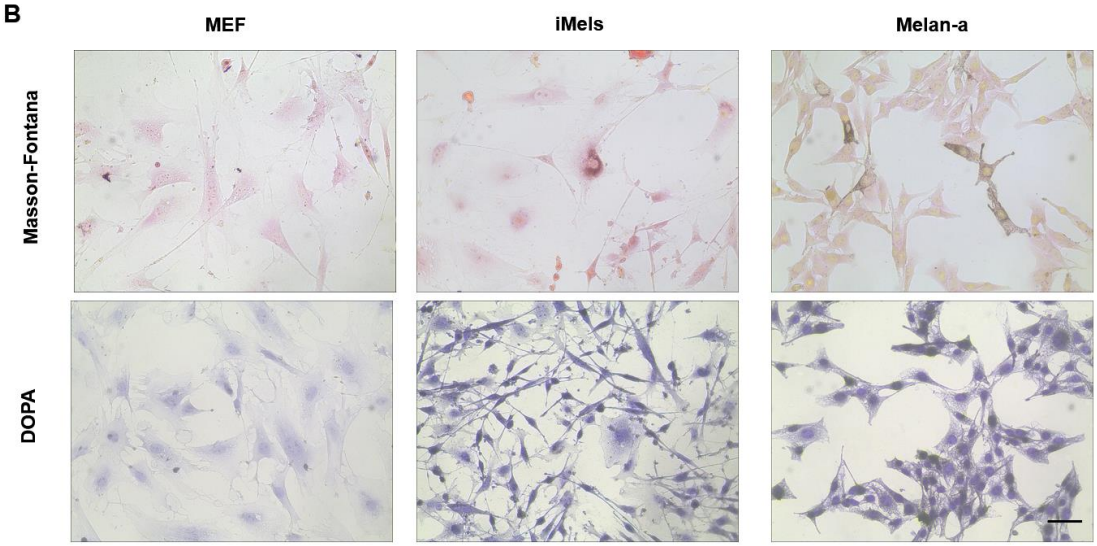
2. It is true that MOI of 30 is a high infection condition, but after our exploration, it does need an MOI of 30 to achieve a relatively high infection efficiency for MEF. This conclusion was verified by flow cytometry (as shown in the figure below). Although the MOI is 30, MEF can still continue to grow (as shown in Figure 2), which may be related to the susceptibility and tolerance of different cells to the virus.



3. According to the reviewer's suggestion, I have added the 6F group as a positive control in Figure 3A. Change as shown below. Figure legend has also been modified in manuscript.



4. According to the reviewer's suggestion, I have added MEF as a negative control and Melan-a cell line as a positive control in Figure 4B. Since the mouse melanocytes we isolated need nourishment from mouse keratinocytes to grow, we chose mouse melanocyte line (Melan-a) as the positive control in staining experiments (as shown in the figure below). Figure legend has also been modified in manuscript.



Minor Concerns:

1. The description of "as few transcription factors as possible" in Summary has been deleted, and I specified the three transcription factors in the last paragraph of Introduction;
2. The manuscript has been polished thoroughly by native English speaker.

To reviewer #2

Comments:

Major Concerns:

1) Throughout the text, especially in the abstract and introduction, it is not clear if the authors want to reprogram melanocytes, or reprogram skin cells to melanocytes, or anything else. Authors should clarify this clearly in the abstract and introduction.

2) The language and grammer should be corrected throughout the text. The error can lead to scientific problems.

3) It is not clear if this protocol has been published and proved in another publication. It is important for the readers to obtain such an information.

Response:

Major Concerns:

1. According to reviewer's suggestion, I have revised the title and emphasized in the Abstract and Introduction that we want to reprogram skin cells into melanocytes.
2. The language and grammar have been corrected throughout the text by native English speaker.
3. This article is the first specific protocol and different from the published paper.
   It is great impact to generate melanocytes through direct reprogramming from fibroblasts. This is not the first report with three transcription factors (3TFs) of Mitf, Sox10 and Pax3 in the field (Yang R, Zheng Y, et al. Nat Commun. 2014;5:5807. PMID: 25510211), since JoVE is a methodology journal, their primary goal is to be a resource for researchers who learn new technologies conveniently. To meet this purpose, we submitted this first specific protocol for JoVE to overcome to the weak points in previous paper and supply a repeatable and practical process of the experiments.
   Although with the 3TFs, our strategies are distinct different from the reports. Such as, 1) Screening. In our protocol, we used a random, fair and reasonable method, and did not prejudge that any transcription factor such as Sox10 had a special role (PMID: 25510211); 2) Transfection. The lentivirus transfection method we used has the characteristics of convenience, strong operability and stability, which is easier to promote and repeat, which different from more complicated Tyrosinase-CreER reporter system to transduce transcription factors, which made it difficult to repeat (PMID: 25510211); 3) Functional identification. We also added the staining representing melanin maturation: Masson-Fontana staining different from PMID: 25510211.
   I have summarized these points in Introduction.

To reviewer #3:

Comments:

In this manuscript, Zhang et al describe their method for inducing melanocytes from mouse embryonic fibroblasts in culture. They test the role of 6 transcription factors and show that 3 are effective at inducing melanocytes from the MEFs. The manuscript is mostly clearly written (some English improvements are necessary) and the method carefully described. They include the essential factors for melanocytes in the reprogramming media. They use several genes (the pigmentation genes Tyr, Tyrp1 and Mlana) to verify that induction has taken place using qPCR and antibody staining as well as DOPA and FM staining to show pigment. In the histological stains the negative and positive controls are missing. I wonder if RNA-seq would be the most appropriate method to show that melanocytes have indeed been induced. The percentage of induced cells needs to be thouroughly discussed.

Responses:

Firstly, the same as previous 2 reviewers' suggestions, the language has been polished thoroughly. Secondly, I have added the negative and positive controls in the histological stains (the same as reviewer #1's major concern 4). Thirdly, RNA-seq is indeed one of the methods to prove melanocytes have been induced, but it also has limitations. RNA-seq's function is similar to more comprehensive qPCR, but RNA-seq cannot detect the function that melanocytes can secrete melanin, so staining is also necessary. Finally, there are also some technical limitations in this protocol, such as the induced efficiency cannot be observed intuitively, perhaps CRISPR-Cas9 gene editing technique can be combined with this protocol to knock in melanocytic genes such as *Tyr* or *Tyrp-1* in initial cells so as to observe the percentage of induced cells during the reprogramming process (I have discussed the issue in Discussion).