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PDF issue: 2026-11-10

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(Citation)
PIGMENT CELL & MELANOMA RESEARCH,32(5):623-633

(Issue Date)
2019-09

(Resource Type)
journal article

(Version)
Accepted Manuscript

(Rights)
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(URL)
https://hdl.handle.net/20.500.14094/90007060

Induced pluripotent stem cells-derived melanocyte precursor cells undergoing differentiation into melanocytes

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Abbreviations: MPCs, melanocyte precursor cells; iPSC, induced pluripotent stem cells.

Summary

Induced pluripotent stem cell (iPSC) technology offers a novel approach for conversion of human primary fibroblasts into melanocytes. During attempts to explore various protocols for differentiation of iPSCs into melanocytes, we found a distinct and self-renewing cell lineage that could differentiate into melanocytes, named as melanocyte precursor cells (MPCs). The MPCs exhibited a morphology distinctive from that of melanocytes, in either lacking the melanosomal structure, or the melanocyte-specific marker genes *MITF*, *TYR* and *SOX10.* In addition, gene expression studies in the MPCs showed high-level expression of *WNT5A*, *ROR2,* which are non-canonical WNT pathway markers, and its related receptor-*TGFβR2*. In contrast, MPCs differentiation into melanocytes was achieved by activating the canonical WNT pathway using the GSK3β inhibitor. Our data demonstrated the distinct characteristic of MPCs' ability to differentiate into melanocytes, and the underlying mechanism of interfacing between canonical and non-canonical WNT signaling pathway.

KEYWORDS: human primary melanocytes, melanocyte precursor cells, induced pluripotent stem cells, WNT signaling, differentiation

Significance

Human primary melanocytes are usually difficult to work with, especially in regard to culture maintenance and this hinders the studies for elucidation of various functions of human melanocytes. We describe a distinctive type of cell, melanocyte precursor cells (MPCs), which are induced pluripotent stem cell (iPSC)-derived and have different characteristics from melanocytes; and, moreover, were converted to melanocytes by activating the canonical WNT pathway using the small amount of GSK3β inhibitor. It is possible that MPCs would be useful tools for studying melanocyte differentiation mechanism under WNT pathway regulation using human pigmentary disorders-specific iPSC.

1 | INTRODUCTION

Epidermal melanocytes play an important role in photo-protection against ultraviolet radiation. Their functional defects can cause various disorders such as vitiligo, albinism and their malignant transformation causes melanoma (Ezzedine et al., 2015; Mort et al., 2015). While commercially available melanocytes were widely used in previous studies, most of them were melanocytes from neonatal foreskins, and we often encounter difficulties in primary culture of melanocytes from adults, especially elderly patients, and their maintenance beyond few passage numbers (Halaban R, 2005). This is due to low mitotic activity in the absence of specific stimuli, such as ultraviolet radiation, phorbol-12-myristate-13-acetate (PMA) or cyclic AMP (cAMP)-inducers (Eisinger and Marko, 1982; Jimbow et al., 1975).

Induced pluripotent stem cells (iPSCs) were reported to be similar to human embryonic stem cells (ESCs) and are generated from adult human fibroblasts by introduction of four genes, Oct3/4, Sox2, Klf4, c-Myc (Takahashi et al., 2007). This strong potential of iPSCs to differentiate into any type of cells, facilitates the generation of iPSCs-derived melanocytes, with relative ease. Indeed, several studies using iPSC technology to convert human primary fibroblasts to melanocytes have already been reported, to explore the pathogenesis of human genetic pigmentary disorders (Mica et

al., 2013; Nissan et al., 2011; Ohta et al., 2011), and investigate each step of melanomagenesis (Larribere and Utikal, 2014) (Larribere et al., 2015). We successfully established protocol for differentiation of iPSCs into melanocytes using feeder-free human iPSCs, which is different from previous protocol using murine-derived feeder cells (Nakagawa et al., 2014). Thereby, we can exclude the effects of other animal cells. In the attempt to explore several protocols for achieving differentiation of feeder-free iPSCs into melanocytes, we found a unique and distinct cell intermediate that rapidly differentiated into authentic melanocytes in a week simply after GSK3β inhibition; it was designated as melanocyte precursor cells, MPCs. We characterized these MPCs as well as the associated factors responsible for their differentiation into melanocytes.

2 | RESULTS

2.1 | Differentiation of feeder-free iPSCs into melanocytes

Although there have been some reports on the protocol for differentiation of on-feeder iPSCs into human melanocytes (Mica et al., 2013; Nissan et al., 2011; Ohta et al., 2011), we attempted to apply their protocol to feeder-free (Ff) iPSCs, in order to obtain cells with higher purity and to exclude mouse-originated feeder cells (Supplemental Materials). We used two Ff-iPSC clones in this study. One is the

conventional iPSC clone 201B7 (Takahashi et al., 2007) and the other is a newly established cell line 21F1. The iPSC line 21F1 showed hES-like cell morphology (Fig. 1a) and expressed pluripotent markers at the protein level (Supplemental Fig. 1a) and the mRNA level (Supplemental Fig. 1b). 21F1 could differentiate into three germ layers (Supplemental Fig. 1c) *in vitro*, and we confirmed that 21F1 was pluripotent and similar to validated normal hPSCs by Pluritest (Muller et al., 2011) (Supplemental Fig. 1d). Moreover, 21F1 had a normal karyotype (Supplemental Fig. 1e).

Floating embryoid bodies (EBs) were cultivated from each Ff-iPSCs set in poly-HEMA (Poly 2-hydroxyethyl methacrylate) coated dishes in iPSCs medium without FGF-2 for two weeks. EBs were plated onto fibronectin-coated dishes, in melanocyte differentiation medium containing SCF, ET3, FGF-2, CHIR, BMP4 and cAMP, as described in Materials and Methods. In 14 days, their morphology had changed into black and spindle-shaped resembling dendritic cells. (Fig. 1a, 1b). We analyzed melanocyte-specific genes, such as *MITF*, *TYR* and *SOX10*, along with iPSCs marker genes, such as *NANOG* and *OCT4*, during the time period from iPSCs differentiation to formation of EBs and into melanocytes. We found high-level expression of the melanocyte-specific markers *MITF*, *TYR* and *SOX10* and low-level expression of *NANOG* or *OCT4* around 28 days after EBs formation (Fig. 1c). Thus,

Ff-iPSCs were able to differentiate into melanocytes upon using the protocol for on-feeder iPSCs. Although differentiated melanocytes were obtained, after about 28 days from formation of EBs, they gradually became slow-growing, with clumped-cell configuration and decreased expression of melanocyte markers (Fig. 1a and 1c). The continuous culture with a mix of stimulating factors was considered to cause damage to the differentiated melanocytes. Therefore, we switched from melanocyte differentiation medium described above, to the simpler M254 medium with human melanocyte growth supplement (HMGS) (life technologies, Carlsbad, CA), which are used to maintain primary cultured human melanocytes; this change in media was carried out at the time point of 28 days after differentiation of Ff-iPSCs, 14 days after EBs formation (Nissan et al., 2011) .

2.2 | Generation of melanocyte precursor cells and their differentiation into melanocytes with GSK3β inhibitor

After shifting to M254 medium with HMGS, some dendritic shaped cells were transformed to distinct polygonal cells with plump cytoplasm without melanin granules and finally almost all of cells in the dish were replaced by such cells. Furthermore, these characteristics of the cells were again changed into that of typical dendritic melanocytes

containing abundant melanin granules, upon addition of 3.0 μM GSK3β inhibitors, CHIR99021, in about seven days (Fig. 1d and Supplemental Fig. 2 movie). These dendritic cells were confirmed as melanocytes based on the fact that they showed strong expression of melanocyte markers in immunocytochemistry (Fig. 1e). Consequently, those characteristic polygonal cells obtained before stimulation with GSK3β inhibitor, were designated as melanocyte precursor cells (MPCs) (Dorsky et al., 1998; Yamada et al., 2013; Yamaguchi et al., 2004). The exact same configuration changes were observed with BIO (CAS 667463-62-9), another GSK3β inhibitor (Sigma, St. Louis, MO), and an inhibitor from another company, CHIR99021 (CultureSure™, Wako, Osaka, Japan) (Supplemental Fig. 3). We designated the melanocytes differentiated from these MPCs as "indirectly differentiated melanocyte" or abbreviated as Ind. Diff. MCs. Furthermore, direct addition of CHIR99021 to differentiating cells 14 days after EB formation, revealed similar cell morphology to that of melanocytes from MPCs (Fig. 1d). These melanocytes which were not obtained through differentiation from MPCs, were termed as "directly differentiated melanocytes" or as Dir. Diff. MCs and indicated as such throughout the text and figures in this article. We also studies whether MPCs could be generated from cells cultured in melanocyte differentiation medium for a longer period of time after EB formation, namely 28 days, when they closely resemble

fully-differentiated melanocytes. The 28-day-stage cells were also successfully converted to MPCs, just like the 14-day-stage cells, and then differentiated into melanocytes by adding GSK3β inhibitors (data not shown). This almost identical reaction indicates that these 14 day-stage cells possess characteristics that are almost entirely exclusive to melanocytes (Fig.1c).

2.3 | Distinctive characteristics of MPCs in comparison to melanocytes

Although differentiated melanocytes with high-level expression of melanocytic markers were obtained from the MPCs, cell morphology of the MPCs was substantially different from that of the melanocytes (Fig. 2a). Under an electron microscope, neither the characteristic melanosome nor the melanin granules that are contained in melanocytes were observed in MPCs. The melanocytic-specific markers *MITF*, *TYR* and *SOX10* were also not barely detected, indicating that the MPCs did not possess the characteristics of melanocytes (Fig. 2b and 2c). In addition, we performed immunocytochemistry study to identify whether the MPCs express Pmel17 (HMB-45), premelanosome protein (Gown et al., 1986), which are relatively specific markers for juvenile melanocytes; MPCs did not show positive staining for HMB-45 in contrast to differentiated melanocytes (Fig. 2d).

2.4 | Non-canonical WNT pathway is up-regulated in MPCs

As melanocyte stem cells have been characterized as expressing PAX3, as well as DCT and FZD4, in the bulge region of the hair follicle (Yamada et al., 2010; Yamada et al., 2013), we performed microarray analysis as follows: MPCs, biological duplicates of differentiated MCs obtained by our protocol (Fig. 1d), human epidermal melanocytes and Ff-iPSCs. Among these cell types, the expression pattern was clearly different. The expression of melanocyte marker genes in the MPCs was distinct from that of either Ff-iPSCs or differentiated melanocytes, including Asian/Caucasian human melanocyte, Ind. Diff. MCs and Dir. Diff. MCs. In the principal component analysis, the MPCs were significantly distant from any other cell types, whereas three cell types of differentiated melanocytes (Asian/Caucasian human melanocytes, Ind.Diff.MCs and Dir.Diff. MCs) positioned relatively close to each other (Fig. 3a). In the hierachical clustering analysis with listed melanocyte differentiation-associated genes, MPCs again showed a different expression pattern compared to that of either the differentiated melanocytes or iPSCs (Fig 3b). We sorted out up-regulated genes (fold change > 10.0) in the MPCs compared with melanocytes group comprising of human epidermal melanocytes, Ind. Diff. MCs and Dir. Diff. MCs, showing that certain genes were up-regulated. Among them we

focused on WNT signaling pathway genes based on the results of ontology analysis, and compared results from MPCs and melanocytes group. Interestingly, non-canonical *WNT5A*, its receptor *ROR2* and its downstream target transcription factor *JUN*, were significantly up-regulated in the MPCs, whereas down-regulation of *LEF1*, downstream of canonical pathway *WNT3A*, was observed although it was not statistically significant (Table 1 and Fig. 3c). Furthermore, TGFβ type II receptor (TGFBR2) was also up-regulated, which is known to play an important role as a targeted receptor of TGFβ (TGFB) signaling, in maintenance of melanocyte stem cells in mouse model (Nishimura et al., 2010). Our microarray data revealed significantly high expression of *TGFBR2* in the MPCs but not in other cells, especially, differentiated melanocytes and human epidermal melanocyte. This suggests that TGFBR2 and ROR2 as well as WNT5A signaling are candidates which control the maintenance of MPCs and differentiation into melanocytes from MPCs is achieved by stimulation of canonical WNT pathway (Fig. 3c).

2.5 | Effect of inhibition of TGFBR2 and ROR on MPCs and melanogenesis

Based on the results showing up-regulation of non-canonical WNT signaling pathway in MPCs (Fig. 3), we finally investigated the characteristics of MPCs that were

changed upon inhibition of TGFBR2 and ROR2. Firstly, phosphorylation of SMAD, an intracellular signal protein transduced by TGFB, was studied without any treatment. Phosphorylated-protein level of SMAD in the MPCs was up-regulated compared with that of directly differentiated MCs and indirectly differentiated MCs (Fig. 4a). With SB431542, which is a TGFBR inhibitor, morphology of MPCs began to change into spindle shaped cells in one week, but dendrite-shaped characteristic of melanocyte morphology remained incomplete at three weeks (Fig. 4b). Secondly, mRNA level of *ROR2* and *FZD2* showed significant reduction by SB431542 treatment in three weeks, whereas the level of the melanocytic markers *MITF* and *TYR* were not altered (Fig. 4c). Thirdly, we studied whether inhibition of ROR2, a non-canonical pathway marker expressed in the MPCs, might exert direct effects on activation of canonical WNT pathway. ROR2 small interfering RNA (siROR2) was administrated in the MPCs along with mock-treatment control. Although it did not manifest the high-level expression as in differentiated melanocytes, the up-regulated expression of *LEF1* was statistically significant. This indicated the inhibition of non-canonical pathway, as *ROR2*-si is partly involved in differentiating MPCs into the melanocytes by activating the canonical WNT pathway (Fig. 4d and Fig. 5). Mice studies have already shown that MPCs might fulfil the criteria for melanocyte stem cells because of their ability for self-renewal as well as

differentiation into melanocytes (Nishimura et al., 2010). The MPCs showed robust cell proliferation in several passages (Fig. 4e), with their viability maintained upon repetitive freeze and thaw manipulations (data not shown), indicating that the MPCs could share some characteristics with those of melanocyte stem cells. Finally, we studied whether ROR2-positive cells are expressed in human skin by immunohistochemical staining. ROR2 positive cells were distributed in the bulge areas of hair follicle (Fig. 4f).

3 | DISCUSSION

Despite difficulties in maintaining melanocytes, remarkable progress in methods for melanocytes culture has been made. Until now, it was recognized that PMA, basic fibroblast growth factor (bFGF), nerve growth factor (NGF) and endothelin-1 (ET-1) were indispensable key growth factors for effective proliferation of melanocytes (Pittelkow and Shipley, 1989); (Jimbow et al., 1975) (Eisinger and Marko, 1982). Even with the application of those agents in the medium, cell proliferation would attenuate along with passages of cells and repetitive freeze and thaw cycles. Therefore, long term culture and maintenance of melanocytes have been difficult, which hinders the progress of studying, especially the human melanocytes. Newly established MPCs demonstrated

good stability and were capable of withstanding repetitive freeze and thaw cycles. We also confirmed the establishment of MPCs from iPSCs derived from four other individuals (data not shown). Thus, once the melanocytes were obtained by single application of GSK3β inhibitor, these differentiated melanocytes were capable of being maintained in the ordinary melanocyte medium.

Although these specific cells, that differentiate into melanocytes by transient treatment with GSK3β inhibitor, are designated as melanocyte precursor cells, another designation of the cells could also be plausible. Melanoblasts, which express *Mitf-M*, *Sox10*, *Pax3*, *Trp-2*, and *Trp-1* in murine skin, could differentiate into melanocyte as well as neurons, glial cells and smooth muscle cells (Motohashi et al., 2009). The MPCs also could differentiate into melanocyte by activation of canonical WNT3A/β-catenin pathway, but were different from melanoblasts because of low-level expression of the melanocyte- and melanoblast-specific markers as well as *DCT*, that are identified in the early stage during differentiation into melanocytes (Fig. 3b) (Mackenzie et al., 1997).

On the other hand, the MPCs could also be designated as melanocyte stem cells, that are populated in bulge areas where hair follicular stem cells reside (Fig. 4f) (Cotsarelis et al., 1990) (Nishimura et al., 2002). The MPCs which were identified in the present study might fulfill the criteria as melanocyte stem cells with their ability for

self-renewal and potential to differentiate into melanocyte (Fig. 4e). Furthermore, the cells designated as MPCs in the present study share various characteristics with previously reported melanocyte stem cells, such as up-regulation of TGFB signaling and its down-stream protein phosphorylation (Nishimura et al., 2010). Our data demonstrated that TGFBR inhibition modulated the morphology of the MPCs and induced a decrease in the expression of *ROR2* and *FZD2*, which are known as WNT5A receptors and are factors of non-canonical WNT signaling pathway. These results indicate that the MPCs are regulated by the non-canonical WNT pathway signaling in addition to the TGFB signaling pathway (Fig. 5). It was already shown that melanocyte stem cells expressed receptors for WNT signaling pathway and activation of the canonical signaling in these cells causes differentiation into pigment-producing melanocytes (Yamada et al., 2013) (Rabbani et al., 2011). The non-canonical WNT5A/ROR2 pathway activation was reported to antagonize the canonical WNT signaling pathway in melan-A positive cells, resulting in inhibition of melanin synthesis (Zhang et al., 2013). Our study showed that the suppression of *ROR2* by siRNA increased expression of *LEF1* which is the key transducer of the WNT signaling pathway and directly induced expression of MITF (Regazzetti et al., 2015); (Saito et al., 2003). This indicates that MPCs' differentiation into melanocytes depend on regulation

of both canonical and non-canonical pathways, namely through the interaction between non-canonical WNT5A/ROR2 pathway and canonical WNT pathway. (Fig. 5). However, the level of *MITF*, a gene functioning simultaneously with *LEF1*, which should have been upregulated by *ROR2*-si inhibition, showed no change, and remained low (Fig. 4d). A previous report has indicated that the upregulation of *SOX10* expression, and not just *LEF1* expression, was required to induce *MITF* expression (Dutton et al., 2001). One explanation for this is that only the inhibition effect of ROR2-si (non-canonical pathway) is not sufficient for differentiation into authentic melanocytes.

The phenomenon of differentiation or reprogramming of somatic cells by induction of WNT signaling pathway, or switch between canonical and non-canonical WNT signaling, is shared in several cell development systems, such as osteogenesis induction (Liu et al., 2009); (Gaur et al., 2005), hematopoietic and lymphopoiesis signaling (Famili et al., 2015) and neural cells development (Pino et al., 2011). It is interesting to note that the morphological development of neural cells was similar, but had opposite pattern, compared with MPCs, in which down-regulation of Wnt5a induced the neural precursors into mature interneurons (Pino et al., 2011). However, it remains to be fully investigated, how yet unidentified specific genes interact with ROR2 in MPCs, as well

as their downstream target genes, to suppress WNT canonical pathway, which should be involved in differentiation of MPCs into melanocytes. These information will reveal the exact site where human MPCs reside.

4 | MATERIALS AND METHODS

4.1 | Differentiation of feeder-free iPSCs into MPCs and melanocytes

Initially, we established feeder-free iPSCs from human fibroblasts (Nakagawa et al., 2014). Human iPSCs were plated on to dishes coated with poly-HEMA (poly 2-hydroxyethyl methacrylate; Sigma, St. Louis, MO) and incubated in primate ES/iPS cell medium (REPROCELL, Yokohama, Japan) for two weeks for embryoid bodies (EBs) formation. Then the EBs were plated on to dishes coated with fibronectin and incubated for two weeks in a medium suitable for melanocyte culture, that is composed of 50% N-2 supplement (Thermo Fisher, Waltham, MA), 30% low glucose DMEM (Thermo Fisher) and 20% MCDB201 (Sigma), with the addition of 50 ng/ml CHIR99021 (Stemgent, Cambridge, MA), SCF (R&D, Minneapolis, MN), 100 nM endothelin-3 (Thermo Fisher), N6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP, Sigma), 20 pM cholera toxin (Sigma), 50 nM TPA (Sigma), 0.05 μM dexamethasone (Sigma), linoleic acid-albumin from bovine serum albumin (Sigma) and

insulin-transferrin-sodium selenite media supplement (Sigma). After spindle-shaped cells with two or three poles and the amount of black melanin granules were proliferated and dominated, the above complex medium with multiple supplements was switched to the simpler Medium 254 (Thermo Fisher) with human melanocyte growth supplement (HMGS, Thermo Fisher). Within seven to ten days of switching the medium, the population of polygonal MPCs began to dominate the cultured cells from melanocyte-like spindle shaped cells. MPCs were capable of being subjected to repeated passage in fibronectin-coated dishes with TrypLE^{TM} Select (Thermo Fisher). Finally, MPCs in Medium 254 with HMGS were drastically differentiated into authentic melanocytes around one week after addition of 3 μM CHIR99021 every two days. The melanocytes thus obtained were also capable for passages in fibronectin-coated dishes with TrypLE^{TM} Select, after reaching confluency.

4.2 | RNA isolation, gene expression profiling, siRNA transfection, and quantitative RT-PCR

The RNA from feeder free-iPS cells, human epidermal melanocytes and differentiated melanocytes were isolated after the cells reached confluence, with Trizol (ThermoFisher). The gene expression profiling was carried out using the SurePrint G3

human GE microarray (Agilent Technologies), according to the manufacturer's protocol. The data were analyzed using the GeneSpring 13.0 software program (Agilent Technologies). The data processing was performed as follows: (i) Threshold raw signals were set to 1.0, (ii) log base 2 transformation was performed, and (iii) the 75th percentile normalization was chosen as the normalized algorithm (http://genespringsupport.com/faq/normalization). The flag setting was performed as follows: The feature is not positive and significant (not detected), not uniform (compromised), not above background (not detected), saturated (compromised), or is a population outlier (compromised). Control probes were removed and only the "detected" probes that were present in at least one sample among all the samples were used for further analysis. The number of probes used for PCA (Fig.3a) and extracting up-regulated genes in the MPCs (Table.1 and Fig.3c) was 31,727. A hierachical clustering analysis for melanocyte differentiation-associated genes was performed using the Euclidean distance and average linkage algorithm.

Micro-array data have been deposited into the Gene Expression Omnibus(GEO) database with Accession No. GSE118227.

ON-TARGET plus SMARTpool siRNA (Horizon discovery, Cambridge, UK) and lipofectamine RNAiMAX (Thermo Fisher) were added to MPCs seeded to 60-80%

confluency, for siRNA transfection. After 48-hour incubation, total RNA of each cells was isolated and subjected to real-time reverse transcriptase PCR (RT-PCR). Two-step quantitative RT-PCR using Universal ProbeLibrary (LightCycler 480 System II; Roche, Mannheim, Germany) was performed. Expression levels were normalized to those of *18S rRNA*.

4.3 | Immunocytochemistry

Cells were fixed in 3% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature. After being washed with PBS, cells were incubated with PBS with 1% BSA for non-specific protein blocking, and then the cells were incubated with diluted anti-MITF antibody (1:50 dilution; Abcam, Cambridge, UK), anti-SOX10 antibody (1:50 dilution; Abcam), anti-TYR antibody (1:50 dilution; Abcam) and anti-PMEL (premelanosome protein) antibody (HMB-45) (1:50 dilution; Abcam) in 1% BSA. After being washed with PBS, the sections were incubated with biotin-conjugated anti-rabbit immunoglobulin G (IgG; Dako, Kyoto, Japan), followed by incubation for 15 min with streptavidin-conjugated fluorescein isothiocyanate (Dako) and counterstain with 4',6-diamidino-2-phenylindole (Southern Biotech, Birmingham, AL).

4.4 | Western blot analysis

Western blotting was performed as described previously (Yogianti et al., 2014). First, 10.0 μg of protein was electrophoresed on 10% sodium dodecylsulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with rabbit polyclonal anti-human pSMAD2 (1:300 dilution; Abcam, Cambridge, UK), rabbit monoclonal anti-human SMAD2 (1:5000 dilution; Abcam), or mouse monoclonal anti-mouse GAPDH (1:5000 dilution; Wako) as the loading control, followed by reaction with horseradish peroxidase (Sigma, St. Louis, MO). The immunoreactive bands were visualized via a chemiluminescence detection system (BIO-RAD, Hercules, CA).

4.5 | Electron scanning microscope

After fixation with 2% glutaraldehyde and osmium tetroxide and dehydration of sample cells, followed by embedding in epoxy resin, specimens were cut on an ultramicrotome and subjected to double staining with lead and uranium (Filgen,Inc., Nagoya, Japan). Specimens were observed through scanning electron microscope (JEM1200EX, JEOL, Tokyo, Japan).

4.6 | Cell proliferation quantification and viability assessment of MPCs

Cell count of MPCs was performed as follows. On day 0, 8,000 cells (=a) were seeded and incubated in M254 medium supplemented with Human Melanocyte Growth Supplement (HMGS) (life technologies). On day 7, cells were counted (=b) and again 8,000 (=a) cells were seeded and incubated for another seven days. On day 14, the cell count was performed (=c) and cell number was calculated as (= b x c/a), followed by seeding of 8,000 (=a) cells. In the same manner, on day 21, the cell count was performed (=d) and cell number calculated as (= b x c/a x d/a), followed by seeding 8,000 (=a) cells and repeat again on day 28.

ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aid 16k10156 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) (CN) and by the Practical Research Project for Rare/Intractable Diseases from the Japanese Agency for Medical Research and Development, AMED (15ek0109028h0002, 16ek0109028h003) (MK, TA and CA). We thank Mari Kohmoto for excellent technical advice and support.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

FIGURE 1. (a) The time-course morphology from feeder-free iPSCs (Ff-iPSCs) to melanocytes. Upon EB formation, they were plated onto fibronectin-coated dishes in melanocyte differentiation medium containing stimulating-factors mix. Bar indicates 100 μm. (b) The features of cell pellets at each time point; day represents after EB formation. HEMCs；human epidermal melanocytes (Asian/Caucasian individual). (c) Time-course expression of melanocyte and iPSCs markers for Ff-iPSCs, EB, differentiated cells and HEMCs. RNA extracted at the indicated time points and

subjected to two-step real-time RT-PCR. The expression of each gene at each time point was normalized to *18S rRNA* expression. (d) The protocol of differentiation from Ff-iPS to melanocyte through formation of melanocyte precursor cell. After EB formation the culture was maintained with the stimulating factors mix (SCF, ET3, FGF-2, CHIR, BMP4, cAMP) for about two weeks, and then, medium was changed to simpler melanocyte maintaining medium with human melanocyte growth supplement. In two weeks, melanocyte precursor cells were formed with markedly robust proliferation. By adding 3.0 μM GSK3β inhibitors CHIR99021 for about seven days, melanocyte precursor cells were differentiated into melanocytes ("indirectly differentiated melanocytes; Ind. Diff. MCs"). "Directly differentiated melanocytes; Dir. Diff. MCs" also could be formed by adding CHIR99021 to the cells featuring bipolar shaped configuration for about seven days. (e) Immunocytochemistry of melanocyte-specific markers expression in differentiated melanocytes obtained from melanocyte precursor cells, along with HEMs as a positive control. Differentiated melanocytes were obtained by stimulation of GSK3β inhibitors CHIR99021 for seven days. Each cell sample was incubated with anti-human monoclonal antibody against MITF, SOX10 and TYR followed by reaction with biotin-conjugated anti-rabbit immunoglobulin G and

streptavidin-conjugated fluorescein isothiocyanate counterstaining with 4',6-diamidino-2-phenylindole. Bar indicates 40 μm.

FIGURE 2. (a) The morphology of the MPCs and human epidermal melanocytes (HMCSs; Asian individual) under phase-contrast microscope. Bar indicates 100 μm. (b) The features under electron microscope of the MPCs, indirectly differentiated melanocytes (Ind. Diff. MCs) and HEMCs. Arrowheads indicate melanosomes. Bar: 400 nm. (c) The expression of melanocyte markers and iPSC markers in the MPCs, Ff-iPSCs, EB, Diff. MCs-1 and HEMCs. RNA extracted at the indicated time points and subjected to two-step real-time RT-PCR. The expression of each gene at each time point was normalized to *18S rRNA* expression. The MPCs-1 and the MPCs-2 were biological duplicate samples. Error bars represent mean ± SD from results of experiments conducted in triplicate. Significant differences among the groups were determined using Student's *t*-test. ***$P < 0.001$, **$p < 0.01$, *$p < 0.05$. (d) Immunofluorescence of melanosome-specific marker expression in the MPCs along with indirectly differentiated MCs (Ind. Diff. MCs). Each cell was incubated with anti-human monoclonal antibody against HMB-45, followed by reaction with biotin-conjugated anti-mouse immunoglobulin G and streptavidin-conjugated

fluorescein isothiocyanate counterstaining with 4',6-diamidino-2-phenylindole. Bar indicates 50 μm.

FIGURE 3. (a) Principal component analysis of microarray data of the MPCs along with Ff-iPSCs, differentiated melanocytes (Dir. Diff. MCs and Ind. Diff. MCs), and human epidermal melanocytes. The percent of variation captured by the first three principal components (PCs) : PC1=48.61% (x-axis), PC2=27.6% (y-axis) and PC3=18.1% (z-axis). (b) Clustering of melanocyte marker genes expression. Heat map of microarray analysis is shown. (c) The expression of specific melanocyte precursor cell marker genes in Ff-iPSCs, the MPCs, directly differentiated melanocytes (Dir. Diff. MCs), indirectly differentiated melanocytes (Ind. Diff. MCs) and human epidermal melanocytes (HEMCs). RNA was extracted points and subjected to two-step real-time RT-PCR. The expression of each gene at each time point was normalized to *18S rRNA* expression. Error bars represent mean ± SD from results of experiments conducted in triplicates are shown. Significant differences among the groups were determined using Student's *t*-test. ***$P < 0.001$, *$p < 0.05$, *N.S*: not significant.

FIGURE 4. (a) The phosphorylation pattern of SMAD2 in the MPCs, directly differentiated melanocytes (Dir. Diff. MCs) and indirectly differentiated melanocytes (Ind. Diff. MCs) without any treatment as determined by Western blotting. (b) The morphological change of the MPCs upon addition of 50 μM SB431542, TGFB receptor inhibitor. During culturing of the MPCs, M254 medium with Human Melanocyte Growth Supplement was used. Bar indicates 100 μm. (c) The expression of melanocyte-specific markers in MPCs upon TGFB receptor inhibition. The expression of each gene in indirectly differentiated melanocytes is shown as reference. Relative expression levels were determined by quantitative reverse-transcription PCR. The expression of each gene at each time point was normalized to 18S rRNA expression. Error bars represent the mean ± SD from results of experiments conducted in triplicates. Significant differences among the groups were determined using Student's *t*-test. (d) MPCs were transfected with siRNAs against target genes (ROR2) or control siRNA immediately after plating, followed by incubation for 48 hr. The expression of *ROR2*, *LEF1*, *MITF*, *TYR*, and *SOX10* transcripts was measured by two-step real-time RT-PCR. Significant differences among the groups were determined using Student's *t*-test. Error bars represent the mean ± SD. $^{**}P < 0.01$, $^{*}p < 0.05$, *N.S*: not significant. (e) Cell growth measurement of MPCs. The MPCs were incubated in M254 medium

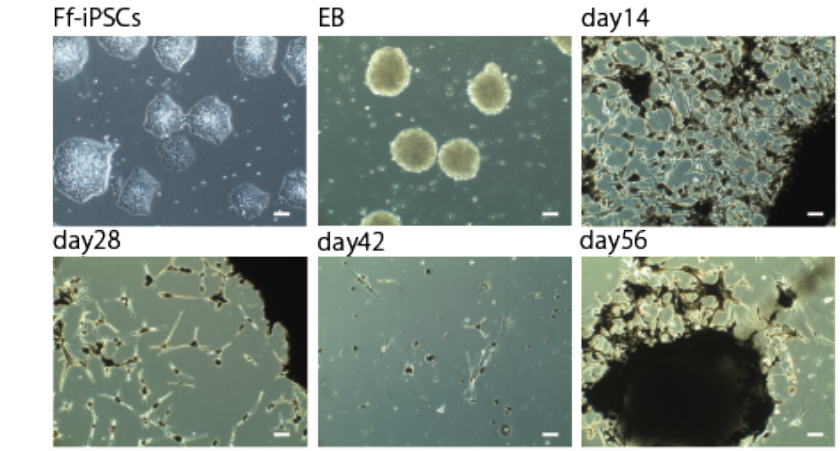
supplemented with Human Melanocyte Growth Supplement and were subjected to cell counts at indicated days. Error bars represent the mean ± SD. (f) Immunohistochemical expression of ROR2 in human skin. Left panel: lower magnification of human hair follicle. Scale bar: 200 μm. Right panel: magnified square of the bulge area of hair follicle; arrowheads indicate ROR2-positive cells. Scale bar: 50 μm.

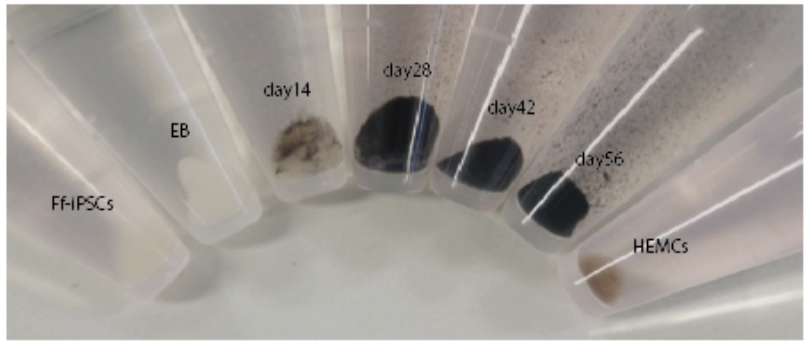
FIGURE 5. Schematic figure of pathways of melanocyte precursor cells (MPCs) into melanocytes differentiation. Differentiation to melanocytes is attributed to an activation of canonical WNT pathway by inhibition of both the GSK3β (indicated with red cascades) and the TGFBR Ⅱ-ROR2 signal, which could be maintaining MPCs in a stable state.

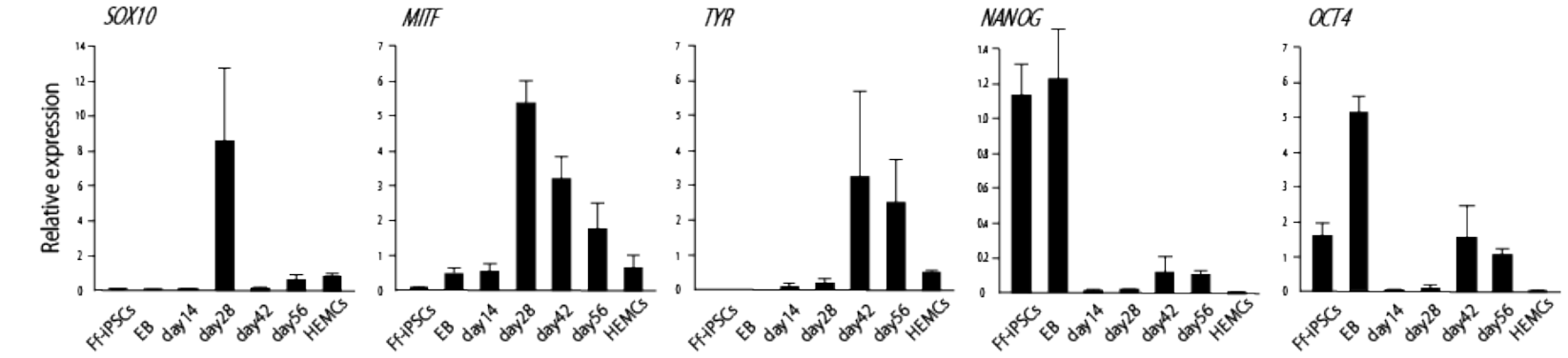
Table 1

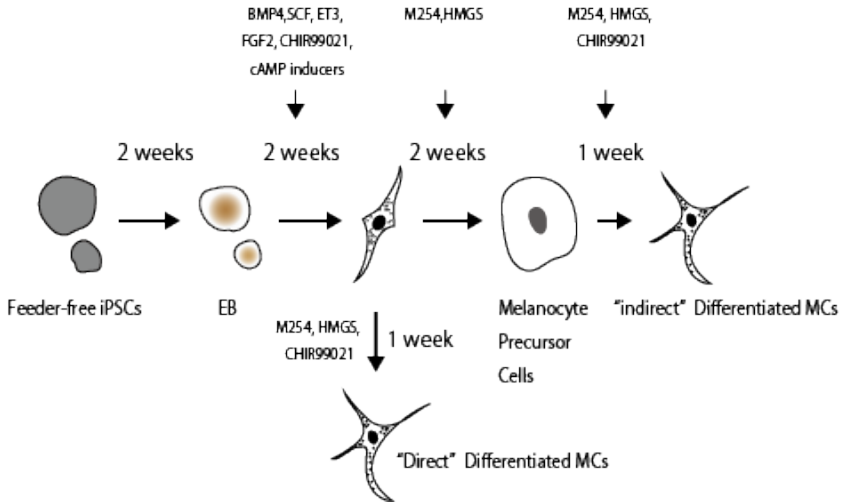
Classification	Gene symbol	Gene name	Ratio (MPCs/melanocyte group)
Ligand			
	WNT5B	Wnt Family Member 5B	109.8
	WNT5A	Wnt Family Member 5A	92.2
Receptor			
	ROR2	Receptor Tyrosine Kinase Like Orphan Receptor 2	197.9
	FZD8	Frizzled Class Receptor 8	21.1
	FZD2	Frizzled Class Receptor 2	13.1
	FZD7	Frizzled Class Receptor 7	12.0
Canonical pathway antagonist			
	SOX17	SRY-Box17	160.0
	SFRP2	Secreted Frizzled Related Protein2	94.9
	PRICKLE1	Prickle Planar Cell Polarity Protein 1	66.9
	SFRP1	Secreted Frizzled Related Protein1	21.5

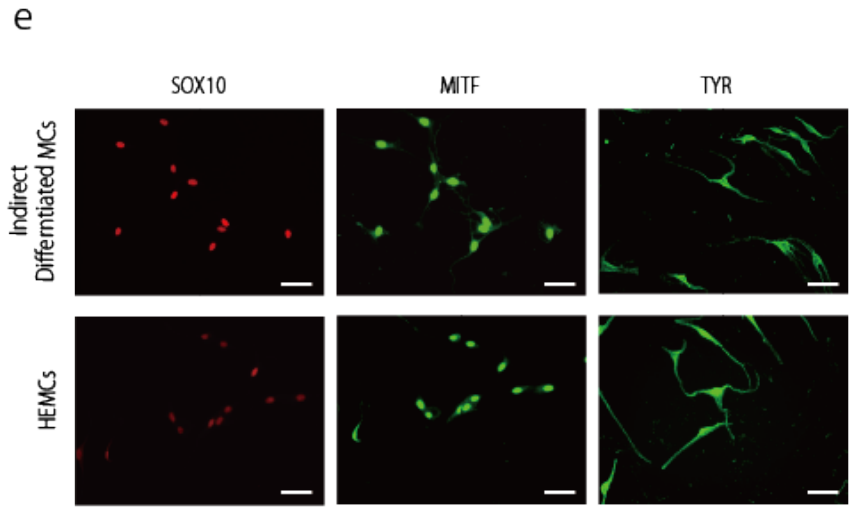
Up-regulated genes in melanocyte precursor cells (MPCs) in WNT pathway genes. Only detected signal intensity in MPCs; in GeneSpring 13.0 software program were extracted and the genes are shown that had >10-fold difference in MPCs compared with average intensity of melanocyte group pool genes, composing of indirectly differentiated melanocytes, directly differentiated melanocytes and human epidermal melanocytes.


a
Ff-iPSCs
EB
day14
day28
day42
day56

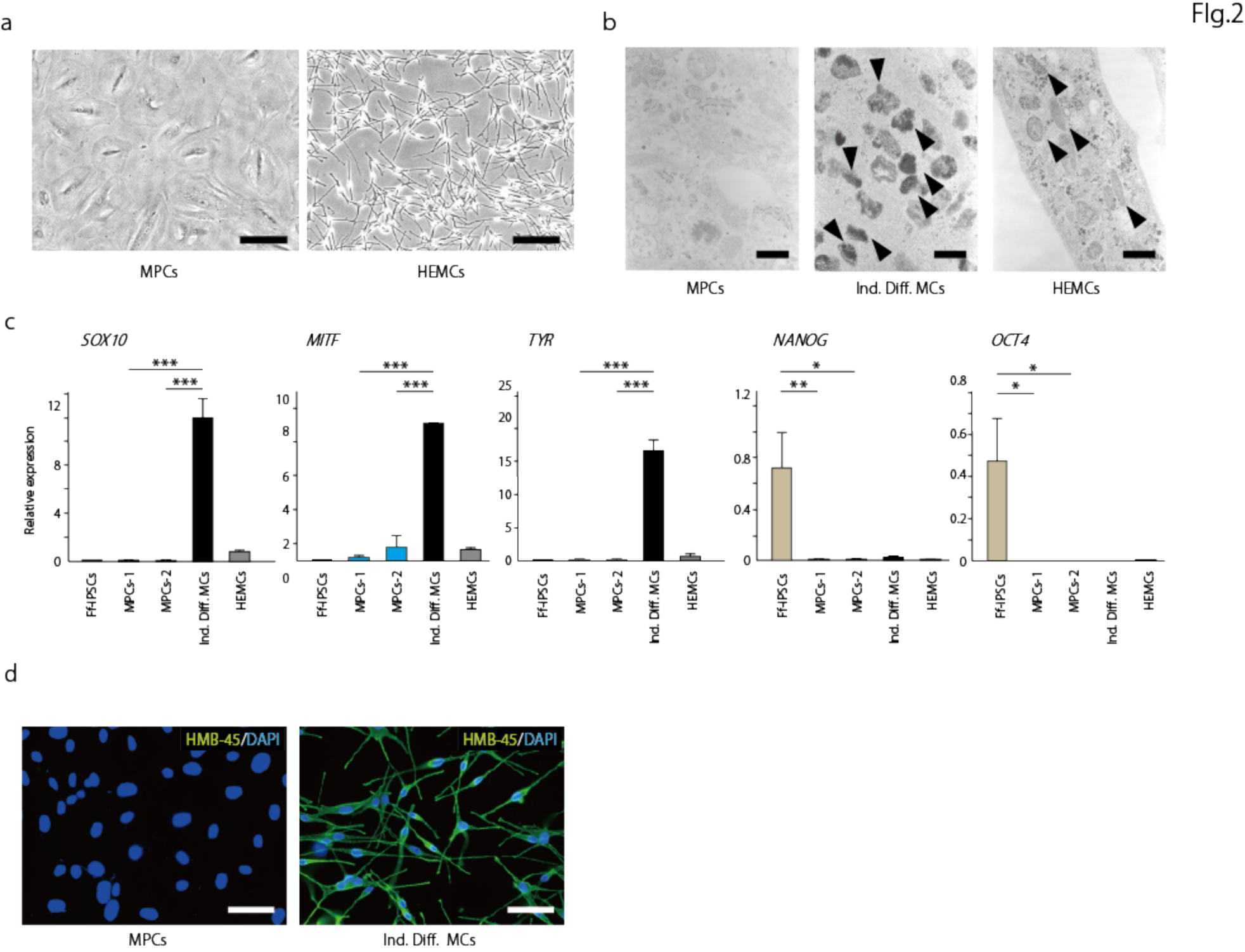

b
Ff-iPSCs
EB
day14
day28
day42
day56
HEMCs


c
SOX10
MITF
TYR
NANOG
OCT4
Relative expression
Ff-iPSCs
EB
day14
day28
day42
day56
HEMCs

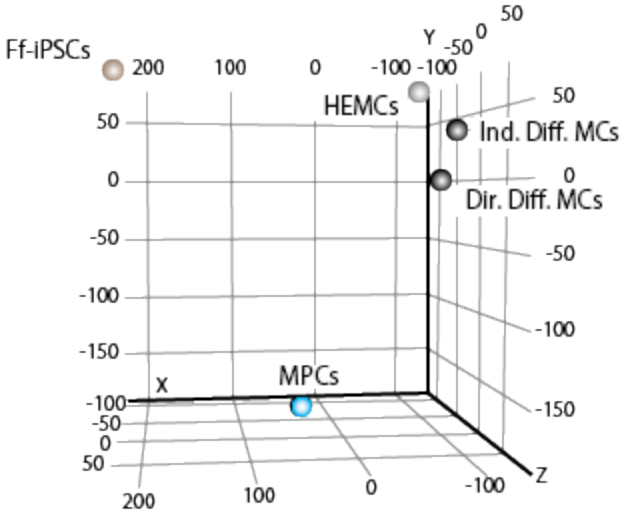

d
BMP4,SCF, ET3,
FGF2, CHIR99021,
cAMP inducers
M254,HMGS
M254, HMGS,
CHIR99021
2 weeks
2 weeks
2 weeks
1 week
Feeder-free iPSCs
EB
Melanocyte
Precursor
Cells
"indirect" Differentiated MCs
M254, HMGS,
CHIR99021
1 week
"Direct" Differentiated MCs


e
SOX10
MITF
TYR
Indirect
Differntiated MCs
HEMCs

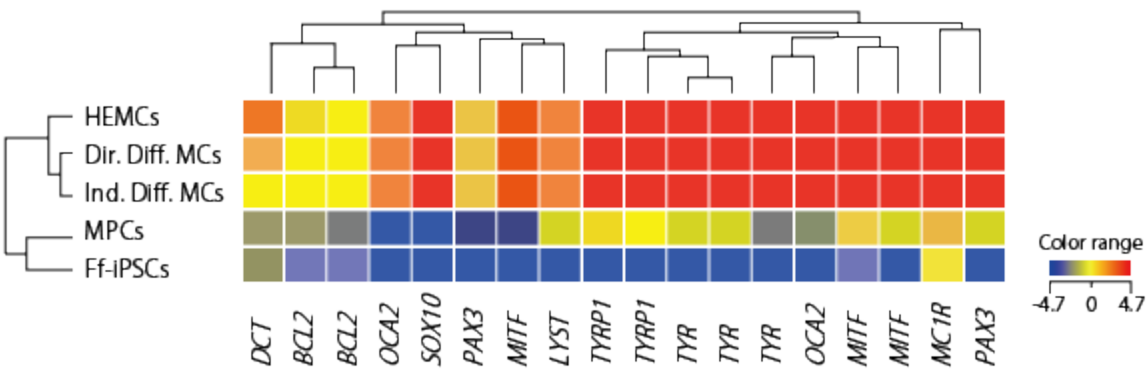
Flg.2


a
MPCs
HEMCs
b
MPCs
Ind. Diff. MCs
HEMCs
c
SOX10
MITF
TYR
NANOG
OCT4
Relative expression
***
**
*
Ff-IPSCs
MPCs-1
MPCs-2
Ind. Diff. MCs
HEMCs
d
HMB-45/DAPI
MPCs
Ind. Diff. MCs

a


Ff-iPSCs
HEMCs
Ind. Diff. MCs
Dir. Diff. MCs
MPCs
X
Y
Z

b


HEMCs
Dir. Diff. MCs
Ind. Diff. MCs
MPCs
Ff-iPSCs
DCT
BCL2
BCL2
OCA2
SOX10
PAX3
MITF
LYST
TYRP1
TYRP1
TYR
TYR
TYR
OCA2
MITF
MITF
MC1R
PAX3
Color range
-4.7
0
4.7

c

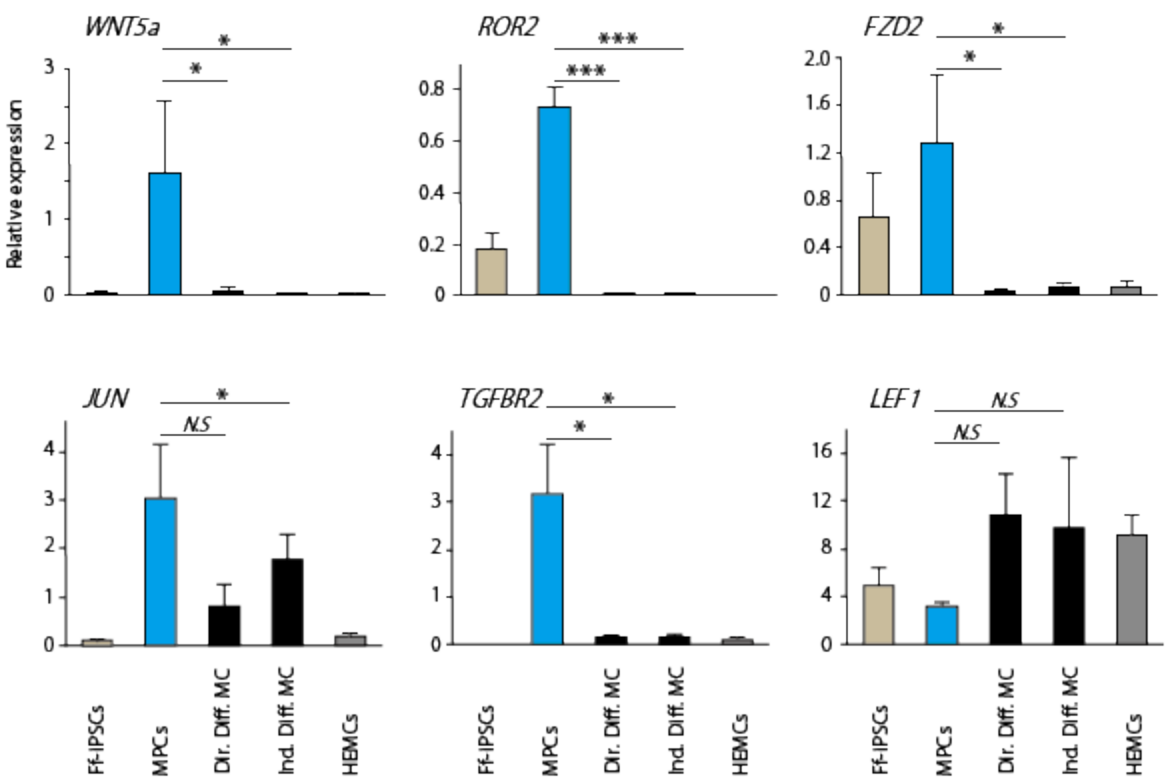

WNT5a
ROR2
FZD2
JUN
TGFBR2
LEF1
Relative expression
NS
Ff-iPSCs
MPCs
Dir. Diff. MC
Ind. Diff. MC
HEMCs

Fig.4

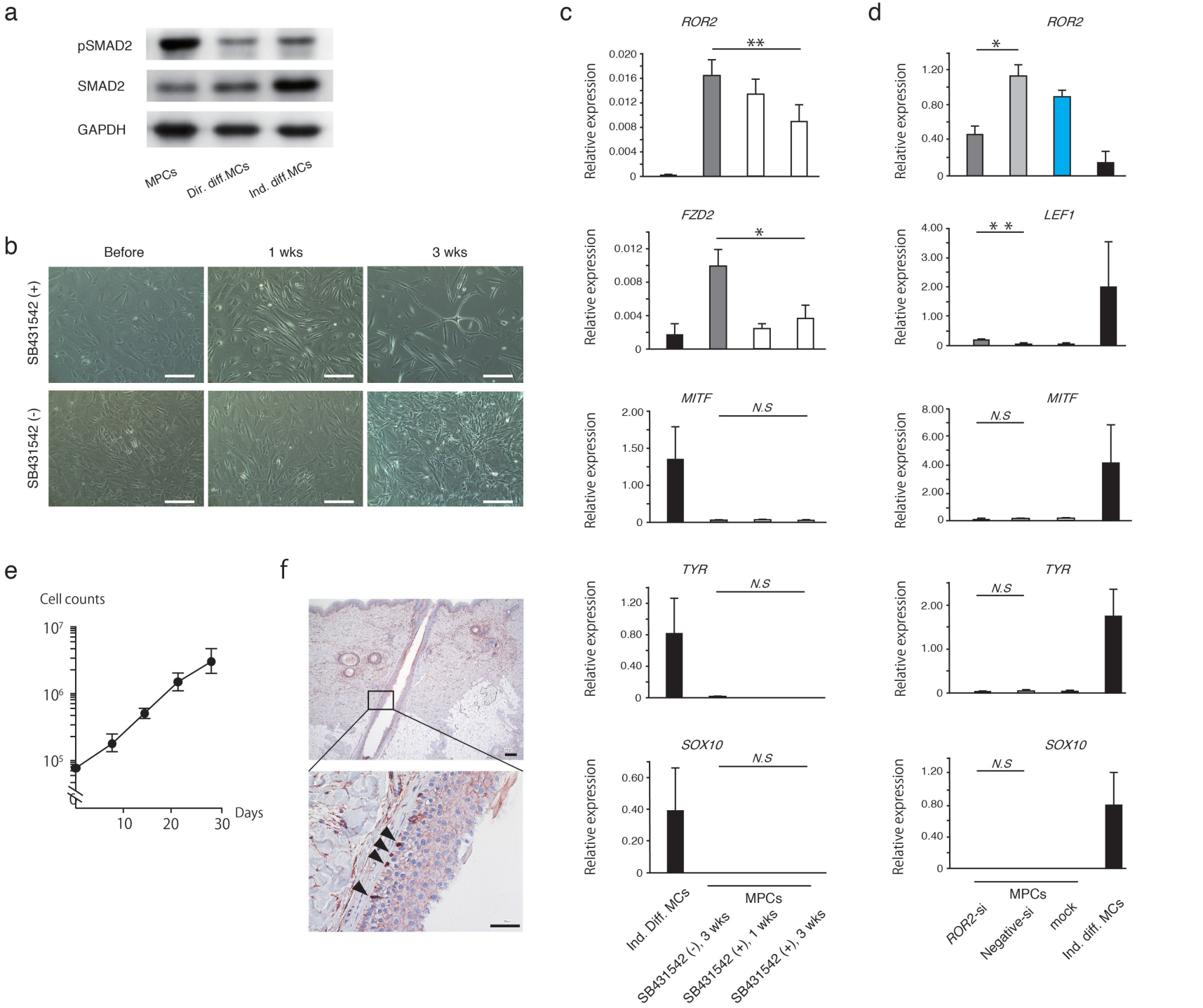
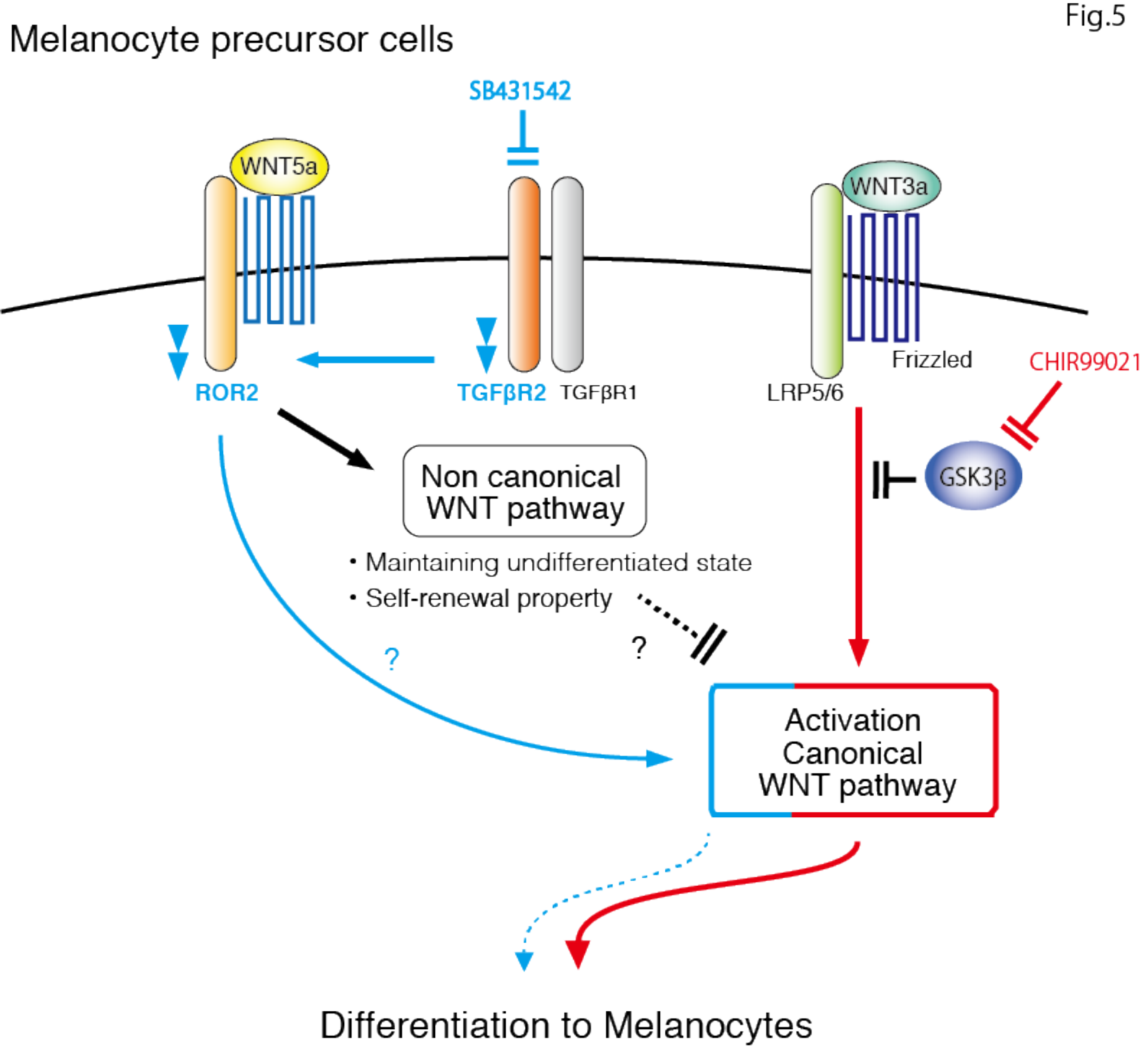

a
pSMAD2
SMAD2
GAPDH
MPCs
Dir. diff.MCs
Ind. diff.MCs
b
Before
1 wks
3 wks
SB431542 (+)
SB431542 (-)
c
ROR2
FZD2
MITF
TYR
SOX10
Relative expression
Ind. Diff. MCs
SB431542 (-), 3 wks
SB431542 (+), 1 wks
SB431542 (+), 3 wks
d
ROR2
LEF1
MITF
TYR
SOX10
MPCs
ROR2-si
Negative-si
mock
Ind. diff. MCs
e
Cell counts
Days
f

Fig.5


Melanocyte precursor cells
SB431542
WNT5a
WNT3a
ROR2
TGFβR2
TGFβR1
LRP5/6
Frizzled
CHIR99021
GSK3β
Non canonical
WNT pathway
• Maintaining undifferentiated state
• Self-renewal property
?
?
Activation
Canonical
WNT pathway
Differentiation to Melanocytes