Dermal Stem Cells Can Differentiate Down an Endothelial Lineage

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In this study, we have demonstrated that cells of neural crest origin located in the dermal papilla (DP) exhibit endothelial marker expression and a functional activity. When grown in endothelial growth media, DP primary cultures upregulate expression of vascular endothelial growth factor receptor 1 (FLT1) mRNA and downregulate expression of the dermal stem cell marker α -smooth muscle actin. DP cells have demonstrated functional characteristics of endothelial cells, including the ability to form capillary-like structures on Matrigel, increase uptake of low-density lipoprotein and upregulate ICAM1 (CD54) in response to tumour necrosis factor alpha (TNF- α) stimulation. We confirmed that these observations were not due to contaminating endothelial cells, by using DP clones. We have also used the WNT1cre/ROSA26R and WNT1cre/YFP lineage-tracing mouse models to identify a population of neural crest-derived cells in DP cultures that express the endothelial marker PECAM (CD31); these cells also form capillary-like structures on Matrigel. Importantly, cells of neural crest origin that express markers of endothelial and mesenchymal lineages exist within the dermal sheath of the vibrissae follicle.

Introduction

A POPULATION OF ADULT STEM cells are located in the dermal papilla (DP) and dermal sheath (DS) of the hair follicle. These cells play an important role during the hair cycle and are capable of directing hair growth [1–5]. Human and rodent dermal stem cells have been found to differentiate down osteogenic, adipogenic, and glial lineages [6–8]. Cells of both the DP and DS can demonstrate potential to repopulate the hematopoietic system in mice [9]. In addition, ovine dermal stem cells have been shown to differentiate into vascular smooth muscle cells, which can display functional properties, such as contractility in response to vasoactive agents and expression of smooth muscle markers [10].

Another repository for multipotent adult stem cells is in the skin epidermis, where populations are located in the interfollicular epidermis, the sebaceous gland, and the hair follicle bulge. It has been hypothesized that under conditions of wound healing these populations of cells can exhibit a high degree of plasticity and are capable of regenerating any of the 3 structures [11]. This is supported by the findings that cells in the bulge region can differentiate into glia, keratinocytes, smooth muscle, and melanocytes. This population of cells has been characterized as expressing nestin, CD34, and lacking keratin 15 expression [12]. Additionally, the

different epithelial stem cell populations have been described to express stem cell markers, including LHX2, SOX9, TCF3/4, LGR5/6, and LRIG1 [13,14] reviewed by Barker et al. [15].

Several different cells of neural crest origin reside in the skin, including melanocytes and cells within epidermal and dermal hair follicle niches [16–18]. Therefore, the cells that compose the DP and DS are largely neural crest derived and this has been defined using a WNT1cre model [16]. The origins of the multipotent adult stem cells located in the bulge region of the follicle are less well defined; however, a sub-population of neural crest-derived stem cells have previously been reported to reside within the follicular bulge region [18]. Neural crest-derived hair follicle stem cells contribute to a large proportion of skin-derived precursors (SKPs). SKPs were first described by [19] and were primarily derived from facial skin. SKPs can differentiate into both neural and mesodermal progeny. Transgenic fate mapping has demonstrated that SKP-forming cells are highly enriched in vibrissae follicles [7]. Interestingly, DP cells can form neurons and glia without the intermediate SKP phase, suggesting that DP cells undergo in vitro reprogramming when removed from their niche. SKPs have been shown to form from trunk back skin [20]. However, these cells are thought to be of glial or melanocytic lineages [16], reviewed by Hunt et al. [21].

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Angiogenesis plays an important role during the hair cycle. During anagen, there is an increase in perifollicular vascularization. During involution and telogen, there is a decrease in these blood vessels, which involves the apoptosis of endothelial cells [22]. The anagen follicle bulb is a sufficient stimulus to promote angiogenesis; however, the DP alone is not sufficient to promote angiogenesis from the surrounding tissue [23]. There is a 4-fold increase in perifollicular vascularization during the anagen phase [22], and this vascularization is associated with vascular endothelial growth factor (VEGF) expression, which has been found to be localized in perifollicular keratinocytes and the outer root sheath (ORS), but not the DP. Transgenic over-expression of VEGF in the ORS increased vascularization, and treatment with the neutralizing VEGF antibody decreased vascularization [22]. Thrombospondin-1, an angiogenesis inhibitor, is upregulated during the catagen and telogen phases of the hair cycle, but not present in midanagen [24]. There is evidence that dermal stem cells may play a role follicle angiogenesis. Cultured DP cells express the vascular endothelial growth factor (VEGF) receptor FLT1 [6]. Furthermore, cultured human DP cells have been shown to have basal levels of nitric oxide production and expression of endothelial nitric oxide synthase (eNOS) [25], functional properties of endothelium. Interestingly, when follicles from transgenic mice with a nestin-driven GFP reporter were transplanted into nude mice, nestin-expressing cells were found to compose nascent blood vessels in the dermis [26].

There is growing evidence that mesenchymal and neuronal lineage cells can differentiate into endothelial cells both in vitro and in vivo. Neuronal stem cells have been shown to differentiate into endothelial cells in coculture conditions in vitro [27], and dedifferentiated adipocytes can also differentiate down an endothelial lineage [28]. Additionally, a population of replicating myocytes in the heart has been shown to differentiate into endothelial cells [29]. The hypothesis for this study is that neural crest-derived cells of the vibrissae follicle have a potential to form cells of an endothelial lineage. We demonstrate that DP cells following primary culture exhibit functional properties of endothelium; that clones of DP cells can demonstrate these properties, thus excluding the possibility of contamination; that cells of the DP competent to exhibit these properties are of neural crest origin; and that these cells can be observed in the uninjured vibrissae follicle.

Materials and Methods

Dissections and culturing rat DP and DS cells

Vibrissae follicle end bulbs were dissected from the miscal pad of PVG rats and WNT1cre/YFP or WNT1cre/ROSA 26R mice, according to methods described [30]. DP and DS were microdissected from end bulbs under a light microscope. Four to 5 explants per well were transferred into 4-well dishes (Nunc) containing either standard media (minimal essential media) (Sigma-Aldrich) or endothelial media (Promocell), both supplemented with L-glutamine, gentamycin, amphotericin-b (Sigma Aldrich), and 20% fetal bovine serum (FBS; Sigma Aldrich). Plates were incubated with no movement for 7 days at 37°C, 5% CO₂, to allow explants to attach and grow out. After a further 7 days, cells were trypsinized and further cultured in 25-cm² flasks. Primary cultures refer to cells grown out from explants before first trypsinization.

Cell culture and dermal clones

DP and DS mixed cultures established from primary explants and clones were grown in minimum essential medium (MEM) supplemented with 20% FBS and 2.5 mM L-glutamine (Sigma-Aldrich) or endothelial media. Human umbilical vein endothelial cells (HUVEC) cells were obtained from Promocell. Rat DP and DS clones were established using methods by Jahoda et al. [6]. The following clones were studied: DP4, DP9, DS1, and DS7. All cells were grown in Nunc plasticware.

RNA extraction and reverse transcriptase–polymerase chain reaction

RNA was extracted from cells using an RNeasy Mini kit, according to the manufacturer's protocol (Qiagen). RNA samples were then DNase treated using the DNA free kit (Qiagen). The quality of RNA was verified by 1 µg of RNA on a 1% agarose gel before cDNA synthesis. One microgram of RNA was converted to cDNA using the high capacity cDNA kit RNA (Applied Biosystems), according to manufacturer's instructions. Prevalidated TaqMan assays obtained from Applied Biosystems were used to detect FLT1, VEGF receptor 2 (KDR), PECAM, von Willebrand factor (vWF), α -smooth muscle actin (α -SMA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Each reaction consisted of 9 µL of the Universal TaqMan Fast Master Mix (Applied Biosystems), 1 µL of primers and probe, and 10 µL of 50 µg/µL cDNA. The relative quantitation method was used in a 96-well plate format. Results were all normalized to an endogenous GAPDH control. Each experiment was performed 3 times, with each reaction in triplicate (7500 FAST real-time PCR; Applied Biosystems). Reverse transcriptase–polymerase chain reaction (PCR) was used to detect expression of SOX2 and CD133. The primer sequences are as follows: SOX2 forward 5'-ACCAGCTCGCAGACCTACAT-3' and reverse 5'-ATGTGTGAGAGGGGAGTGT-3'. CD133 forward: 5'-GCTGGACGGGAGCGAGATGTTA-3' and reverse 5'-GGCCACAAAGCCAAACGCGA-3. The control, GAPDH, forward 5'-ATGGCCTACATGGCCTCCAAGG-3', and reverse 5'-AGGCCCTCCTGTTGTTATGGG-3'. Reactions were separated on an agarose gel and DNA was stained with EtBr.

Immunohistochemistry on frozen sections

Rat vibrissae follicle end bulbs were isolated from the mystacial pad of PVG rats. End bulbs were embedded in cryo embedding medium (OCT) (Raymond A. Lamb Limited), snap-frozen in liquid N₂, and stored at –80°C. For WNT1cre/YFP mice, whole follicles were fixed in 4% PFA and then cryoprotected in 30% sucrose before embedding in OCT. Seven micrometer sections were cut at –21°C using a cryostat (CM3050; Leica) onto SuperFrost slides (Menzel-Glaser). Slides were air-dried, washed with phosphate-buffered saline (PBS)/0.4% Triton-X, blocked in 10% of the appropriate species serum, and incubated with the primary antibody overnight at 4°C in PBS/0.4% Triton-X. After this, 3 washes with PBS were carried out before incubating with the secondary antibody for 45 min at room temperature in the presence of 0.5 µg/mL 4',6-diamidino-2-phenylindole. Slides were washed a further 3 times before mounting with the Vectashield (Vector Laboratories) and examining under a fluorescence microscope (Axio

Imager.M1; Zeiss), or for WNT1cre experiments a confocal microscope was used (SP5; Leica).

Antibodies

The following primary antibodies were used: anti-versican antibody (DSHB, Iowa University), anti-PECAM (clone Mec13.3) from BD Biosciences, anti- α -SMA (ab5694), anti-GFP (ab6662), and anti-vWF (ab6994) were all from Abcam. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-PECAM (ab25563) and ICAM (ab23835) flow cytometry antibodies were also from Abcam. Secondary antibodies included goat anti-rabbit IgG Alexa Fluor 488 and donkey anti-mouse IgG Alexa Fluor 594 from BD Biosciences. Both primary and secondary antibody solutions were made up in a 1% donkey serum and 1% goat serum-PBS solution.

Matrigel tube-forming assays

Ninety-six-well plates containing 100 μ L of Matrigel (BD Biosciences) were prepared. Matrigel was allowed to set at 37°C for 30 min. DP or DS clones were seeded onto Matrigel at a density of 4×10^4 cells per well in either standard 20% FBS media or endothelial growth media (Promocell).

Flow cytometry for ICAM and PECAM expression in primary cultures and clones

Rat DP, DS, and dermal clones were plated at 1×10^5 cells per well in 9.5-cm² culture area 6-well dishes (Thermo Scientific Nunc) and incubated for 2 days. Differentiated cells were grown in endothelial media for at least 5–7 days before experiments. For ICAM induction experiments, cells were treated with 5 ng/mL rat TNF α (R and D Systems) for 5 h before harvesting for flow cytometry. The levels of PECAM expression were examined in unstimulated cells grown in either standard or endothelial media. For harvesting, cells were detached from plates by brief trypsinization before centrifugation at 300 g for 10 min. Cells were resuspended in 90 μ L of ice-cold PBS and stained with 10 μ L of mouse anti-rat ICAM-FITC or PECAM-FITC-conjugated antibody. Cells were washed using a fluorescence-activated cell-sorting (FACS) lyse machine (BD Biosciences) before running on the FACS Calibur (BD Biosciences) flow cytometer. Dead cells were excluded by propidium iodide (PI) staining with 1 μ g/mL PI added to samples 5 min before running on the flow cytometer. Experiments were performed in triplicate.

Acetylated DiL-low-density lipoprotein uptake assay

Rat DP, DS, and dermal clones were plated at 4×10^4 cells per well in a 24-well dish (Nunc). Differentiated cells were grown in endothelial media for 5–7 days before experiments. Experiments were carried out as described in [31]. Briefly, stained cells were incubated with media supplemented with 10 μ g/mL acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (ac-DiL-LDL) (Biomedical Technologies, Inc.) for 4 h. The media were then removed and adherent cells were washed 3 times with PBS before trypsinization. Trypsin was neutralized with standard media and cells were pelleted by centrifugation, and resuspended in PBS before running on the flow cytometer. Experiments were performed in triplicate.

WNT1cre mice

To create WNT1cre/YFP experiments, WNT1cre mice [32] were crossed with R26R-EYFP reporter mice [33] to produce litters in which neural crest cells expressed enhanced yellow fluorescent protein (eYFP). For WNT1cre/ROSA26R experiments, WNT1cre mice were crossed with ROSA 26R mice [34] to produce litters in which neural crest cells have the β -galactosidase activity [35]. DP and DS cultures were prepared as described above and hair follicles were embedded in OCT for immunohistochemistry (IHC) (also described above). Cultures were assayed for the β -galactosidase activity using fluorescein di(β -D-galactopyranoside) (FDG) staining and flow cytometry [36,37]. Cultures were briefly trypsinized and pelleted at 300 g. Cells were resuspended in 100 mL of PBS and 2.5 mL of allophycocyanin (APC) labeled rat anti-PECAM or APC-isotype control were added. Samples were kept on ice for 20 min before washing with the FACS lyse machine. Cells were resuspended in 100 μ L of a staining solution containing PBS, 10 mM HEPES, and 4% FBS at pH 7.2. A 20 mM stock of FDG (Sigma Aldrich) made up in dimethyl sulfoxide (Sigma Aldrich) was diluted further to 2 mM in deionized water. Both the 2 mM FDG solution and the cells were incubated at 37°C for 10 min before 100 μ L of FDG was added to the cells and incubated for 2 min at 37°C. The cells were then further diluted with ice-cold 1,800 μ L of staining solution supplemented with 1 μ g/mL PI (Sigma-Aldrich) before running on the flow cytometer. Dead cells were excluded through staining with PI and FDG fluorescence was assessed in the FITC (FL-1) channel.

X-Gal staining of in vitro Matrigel assays

Matrigel assays were fixed using 100 μ L of 4% paraformaldehyde for 10 min. The gels were carefully washed 3 times with X-GAL rinse solution (PBS supplemented with 2 mM MgCl₂, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide (Sigma Aldrich) before incubating with 50 μ L of X-GAL stain, which is composed of the rinse buffer supplemented with 1 mg/mL X-GAL (BDH) for 24 h at room temperature. Results were examined using a light microscope. Experiments were performed in triplicate.

Results

Expression of vascular endothelial markers in primary cultures

Primary DP and DS were cultured in vitro, in endothelial or standard media and the levels of endothelial marker mRNA expression were measured using quantitative real-time PCR (Fig. 1). The expression of KDR, vWF, and PECAM mRNA decreased or remained the same in both DP and DS cultures grown in endothelial media compared to the standard media cultures (Fig. 1a–c). However, the expression of FLT1 mRNA increased 10.8 ± 5.2 -fold in DP cultures grown in endothelial media compared to the other cell types (Fig. 1d). The levels of FLT1 mRNA expression were significantly higher in the DP primary cultures grown in endothelial media compared to the standard DP cultures or DS cultures grown under both conditions [$P < 0.0001$, 1-way analysis of

FIG. 1. Endothelial marker expression profile of primary cultures. Levels of endothelial marker mRNA expression determined by quantitative real-time polymerase chain reaction (qRT-PCR) in primary cultures of dermal papilla (DP) and dermal sheath (DS) cells (a) KDR, (b) von Willebrand factor (vWF), (c) PECAM, (d) vascular endothelial growth factor receptor 1 (FLT-1), and (e) α -smooth muscle actin (α -SMA). Cultures were grown in standard media (DP and DS) or endothelial media [DP(E) and DS(E)]. Levels of expression were normalized to glyceraldehyde 3-phosphate dehydrogenase and expressed relative to the DP standard culture. mRNA extracted from the rat aorta was used as a positive control.

variance (ANOVA), Tukey's]. The levels of expression of the dermal marker α -SMA [38] decreased significantly in both the DP and DS cultures in endothelial media compared to standard media controls for both DP and DS cultures ($P < 0.001$, 1-way ANOVA, Tukey's) (Fig. 1e). Additionally, we examined the mRNA expression of pluripotency markers SOX2 and CD133 in the DP and DS standard cultures. Both DP and DS cells expressed SOX2. DP, but not DS cells also showed strong expression of CD133 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Taken together, these data suggest that culture in endothelial media may facilitate endothelial commitment from dermal cells. To address this, we investigated the functionality of these cell types.

Endothelial functional activity of DP and DS primary cultures

To determine if DP and DS primary cells have functional characteristics of endothelial cells, we used 3 well-characterized in vitro functional assays of endothelial cells: the ability to form capillary-like structures on Matrigel; the capacity for low-density lipoprotein uptake; and the regulation of ICAM in response to TNF α . First, DP and DS primary cultures grown in standard or endothelial media for 7 days were seeded on Matrigel and incubated for 18 h. Neither DS cells grown in either media nor DP cells grown in standard media showed any capacity to form capillary-like structures (data not shown). However, DP primary

FIG. 2. Primary culture endothelial functionality. **(a)** In vitro Matrigel assays with human umbilical vein endothelial cells (HUVEC) (positive control), fibroblasts (negative control), DP and DS primary cultures both grown in endothelial media. The measurement bar on all 4 pictures represents 100 μ m. **(b)** Levels of ac-DIL-LDL uptake in DP and DS primary cultures determined by flow cytometry in cultures grown in standard media (DP and DS) and endothelial media [DP(E) and DS(E)]. **(c)** Histograms showing the levels of surface ICAM1 expression after tumor necrosis factor alpha (TNF α) stimulation in DP and DS primary cultures determined by flow cytometry. Color images available online at www.liebertpub.com/scd

cells grown in endothelial media formed a network of capillary-like structures entirely consistent with those seen in the HUVEC-positive control (Fig. 2a). To further examine the endothelial functionality of DP and DS primary cultures, the capacity for LDL uptake was assessed using ac-DIL-LDL. There was a significant increase in ac-DIL-LDL uptake in both DP and DS cultures grown in endothelial media compared to those grown in standard media [$P < 0.01$, 2-way analysis of variance (ANOVA)], but no difference between the DP or DS cultures ($P = 0.63$, 2-way ANOVA) (Fig. 2b). Induction of ICAM-1 by TNF α is a key

characteristic of cells of endothelial lineage. DP and DS cells showed a significant increase in ICAM-1 expression when cultured in the endothelial medium ($P = 0.05$, 2-way ANOVA) (Table 1 and Fig. 2c).

Endothelial expression and function in dermal clones

To exclude the possibility that the changes in marker expression and activity in endothelial activity assays was due to endothelial cell contamination, dermal clone cell lines established by [6] from single cells of rat DP or DS cultures

TABLE 1. SUMMARY OF FLOW CYTOMETRY RESULTS ON THE RAT DERMAL PAPILLA AND DERMAL SHEATH PRIMARY CULTURES AND THE RAT DERMAL PAPILLA AND DERMAL SHEATH CLONES GROWN IN STANDARD OR ENDOTHELIAL MEDIA AND TESTED FOR ICAM (CD54) UPREGULATION AFTER TREATMENT WITH TNF α

Cell type/line	% Induction of ICAM after TNF α stimulation	
	Standard media	Endothelial media
DP primary culture	-2.0 $\pm$ 7.3	13.6 $\pm$ 5.0
DS primary culture	0.6 $\pm$ 3.4	8.7 $\pm$ 4.8
DP4	59.2 $\pm$ 11.7	78.8 $\pm$ 2.2
DP9	52.5 $\pm$ 3.0	51.0 $\pm$ 2.7
DS1	49.4 $\pm$ 12.4	45.6 $\pm$ 7.0
DS7	61.5 $\pm$ 3.8	48.3 $\pm$ 9.8

TNF α , tumour necrosis factor alpha; DP, dermal papilla; DS, dermal sheath.

were tested. The levels of endothelial marker mRNA expression were examined in 4 dermal clones: DP4, DP9, DS1, and DS7 when grown in endothelial media for 7 days. Similar to the primary cultures, the levels of KDR, vWF, and PECAM all decreased or stayed the same when DP and DS clones were grown in endothelial media compared to standard media (Supplementary Fig. S2a–c). Interestingly, the levels of FLT1 mRNA expression increased 5-fold in the DP4 clones after 7 days in endothelial media compared to standard media. The levels of FLT1 mRNA expression were significantly different between the different clones, with the DS clones having significantly higher levels of FLT1 mRNA expression compared to the DP clones ($P < 0.001$, 1 way ANOVA, Tukeys). However, the DP4 clone was the only one to show a significant increase in FLT1 mRNA expression in response to endothelial growth media ($P < 0.01$, t -test) (Fig. 3a). SOX2 mRNA was expressed in all of the DP and DS clones. CD133 was only expressed in the DP9 clones (Supplementary Fig. S1).

The tube-forming ability of all 4 dermal clones was tested on Matrigel (Fig. 3b). Two of the dermal clones (DP4 and 9) formed a network of capillary-like structures after 18 h, which were very comparable to that of the positive endothelial control (Fig. 3b). Consistent with the primary cultures, none of the DS clones formed a network of capillary-like structures (Fig. 3b). Dermal clones were measured for ac-DIL-LDL uptake. All clones showed a significantly greater capacity for ac-DIL-LDL uptake than control cells, although the different clones showed this to varying degrees (13.7%–42.4%). Culture in endothelial media had little effect on 2 of the clones, but DP4 showed a significant increase in ac-DIL-LDL uptake, with values which were ~62% of that of the positive endothelial control ($P < 0.001$, 1-way ANOVA, Tukey's) (Fig. 3c). All 4 of the unstimulated dermal clones

showed strong and significant upregulation of ICAM1 expression on TNF α stimulation ($P < 0.001$, 1-way ANOVA) (Table 1 and Fig. 3d). This effect was enhanced in the DP4 clone by growing in endothelial media (Table 1). These data indicate that cells of the DP can exhibit properties very similar to cells of endothelial lineage following culture in vitro. Importantly, previous studies on the DP4 cells have clearly demonstrated that these cells are of a mesenchymal lineage and are capable of differentiating down both an adipogenic and osteogenic lineage [6]. However, they do not address where such cells are within the structures of the DP in vivo, or their developmental origin. To answer this, we used a transgenic model, which has been widely used for fate mapping of neural-crest derived cells, WNT1-cre [16,18,20].

Neural crest origins of cells expressing mesenchymal and endothelial markers

Vibrissae follicles from the WNT1cre-YFP mice were examined by immunofluorescence to determine whether cells that express endothelial markers can be of neural crest origin. We first examined the endothelium of vessels from coronary arteries for expression of vWF, PECAM, and YFP; no endothelial cells were found to express YFP (data not shown). This confirms that endothelial cells are generally not of neural crest origin. This is consistent with a previous study, which showed that endothelium in the skin of the face was not of neural crest origin [39]. As previously reported by [16,18,20], cells of neural crest origin are located in the DP and DS (Supplementary Fig. S3). In the DS, we have identified a small population of cells that express both PECAM and vWF and are derived from the neural crest (Fig. 4). In anagen, this population of cells is located as a distinct single cell layer in the DS. Neural crest cells in this region also costain for KDR (Supplementary Fig. S3). To confirm that these cells are within the DS, we costained with the mesenchymal marker α -SMA and identified cells that are of neural crest origin and express both PECAM and α -SMA (Fig. 5). Interestingly, the population of neural crest-derived cells that costain for endothelial markers is less in the catagen follicles than in anagen follicles (Figs. 4 and 5 and Supplementary Fig. S3). The population of dermal, neural crest-derived endothelial cells resides within the DS, which also costains for mesenchymal markers and endothelial markers in rat vibrissae follicles (Supplementary Fig. S4).

To examine the activity of neural crest-derived DP and DS cells in culture, DP and DS primary cultures were established from the follicles of WNT1cre/YFP mice and ' ROSA26R mice and grown out in standard or endothelial media in vitro. Cultures grown from WNT1cre/YFP explants for 7 days were stained for PECAM expression (Fig. 6a). Cells costaining for PECAM and YFP were observed to grow out from the DP explants. In addition, the overall levels of PECAM expression were

FIG. 3. Dermal clone expression and endothelial functionality. (a) Levels of FLT1 mRNA expression determined by qRT-PCR in the DP and DS clones. Cells were grown in either standard media (DP4, DP9, DS1, and DS7) or endothelial media [DP4 (E), DP9 (E), DS1 (E), and DS7(E)]. (b) Tube-forming ability of the DP and DS clones cultured in endothelial media on Matrigel. Measurement bar for DP4 and DP9 represents 50 μ m, and bar on DS1 and DS7 represents 100 μ m. (c) Levels of ac-DIL-LDL uptake in DP and DS clones grown in standard or endothelial media (E). HUVEC-positive control and fibroblast-negative control. (d) Histograms showing the levels of surface ICAM1 expression after TNF α stimulation in DP4 and DP9 clones determined by flow cytometry (results summarized in Table 1). Color images available online at www.liebertpub.com/scd

FIG. 4. In vivo expression of PECAM and vWF in WNT1cre/YFP mice. **(a)** Immunohistochemistry on the end bulb of a WNT1cre/YFP vibrissae follicle (anagen phase) stained for YFP (green), PECAM (purple), and vWF (red). Measurement bar represents 25 μ m. Individual images for **(b)** YFP, **(c)** PECAM, **(d)** vWF, and **(e)** 4',6-diamidino-2-phenylindole (DAPI) nuclear stain. The white arrows indicate cells within the dermal sheath that costain for YFP, PECAM, vWF, and DAPI. Color images available online at www.liebertpub.com/scd

examined in the primary cultures using flow cytometry. There was a significant difference in PECAM expression between the DP and DS primary cultures grown in standard or endothelial media ($P=0.03$, 1-way ANOVA), the levels of PECAM expression were significantly higher in the DP cells grown in endothelial media than the other DP standard media and DS cultures ($P<0.05$, 1-way ANOVA, with contrasts) (Fig. 6b).

The neural crest origins of the WNT1cre/ROSA26R DP cultures were determined using FDG staining for the β -gal activity using flow cytometry. The levels of FDG staining were measured in the DP cultures grown in endothelial media, with strict gates applied to exclude background. Re-

sults were compared to the WNT1cre-negative ROSA cultures. The levels of FDG staining in the WNT1/cre-positive cultures were $7.5\% \pm 4.0\%$, which was significantly higher than in the WNT1/cre-negative cultures $0.6\% \pm 0.4\%$ ($P=0.02$, t -test). In the total cultures $1.9\% \pm 0.6\%$ of cells stained positive for PECAM and FDG (Fig. 6b), significantly higher ($P<0.01$, t -test) than the levels observed in the WNT1cre-ve controls ($0.3\% \pm 0.3\%$). Mouse DP cultures grown in endothelial media for 7 days before in vitro Matrigel assays formed networks of capillary-like structures after 18h, consistent with studies described above. When stained for the β -gal activity, these capillary-like structures

FIG. 5. In vivo expression of PECAM and α -SMA in WNT1cre/YFP mice. Immunohistochemistry on the end bulb of a WNT1cre/YFP vibrissae follicle (a) anagen and (b) catagen phase, stained for YFP (green), PECAM (purple), α -SMA (red), and DAPI nuclear stain (blue). Melanocytes surrounding the DP in the collagen capsule are also derived from the neural crest and also stain for YFP. Measurement bar represents 25 μ m. (c) Cells within the DS that costain with YFP, PECAM, and α -SMA. Individual images showing: (d) YFP, (e) α -SMA, (f) PECAM, and (g) DAPI nuclear stain. Measurement bar represents 10 μ m. White arrow denotes cells that costain for YFP, PECAM, and α -SMA. Color images available online at www.liebertpub.com/scd

FIG. 6. Neural crest-derived DP cells in cultures. **(a)** Immunohistochemistry on WNT1cre/YFP DP cultures grown in endothelial media for 7 stained for **(i)** YFP (green), **(ii)** PECAM (red), **(iii)** light-phase image, and **(iv)** an overlay of all 3 pictures. **(b)** Staining for FDG and PECAM on DP cultures set up from the WNT1cre/ROSA 26R mice and grown in endothelial media. Negative control is a DP culture set up from negative crosses from the same litter. Cells were stained with FDG and isotype APC control. **(c)** **(i)** DP cultures from WNT1cre-negative/ROSA 26R cells grown in endothelial media and grown on Matrigel, and then stained with X-GAL. **(ii)** DP cultures from WNT1cre-positive/ROSA 26R cells grown in endothelial media and grown on Matrigel stained with X-GAL. The measurement bar is equivalent to 25 μ m. APC, allophycocyanin. Color images available online at www.liebertpub.com/scd

demonstrated consistent expression of the reporter gene, consistent with the neural crest origin of these cells (Fig. 6c).

Discussion

A population of dermal stem cells have previously been described to reside within the DP and DS of the vibrissae follicle. The rodent vibrissae anagen DP is composed of cells from a neural crest origin and are enriched for SKP-forming cells [7]. We have previously shown that both rodent and human DP and DS cells have the plasticity to differentiate down adipogenic, osteogenic, and glial lineages [6]. Functionally, cells of the DP can reconstitute the mouse hematopoietic system; function as vascular smooth muscle cells, and

express VEGF during anagen to promote interfollicular angiogenesis [9,10,22]. In this article, we have described the ability of DP cells to differentiate down an endothelial lineage and exhibit similar functional characteristics to endothelial cells in *in vitro* functional assays. Data for DP primary cultures suggest that majority of cells in primary cultures have the ability to function as endothelial cells when exposed to angiogenic signals *in vitro* and DS cells do not exhibit the same plasticity. However, it should be noted that mesenchymal stem cells, monocytes, and macrophages have previously been found to form tube-like structures on Matrigel [40,41]. This indicates that although the *in vitro* Matrigel reflects the potential *in vivo* activity, it is not a definitive assay for endothelial function. Our conclusion that DP cells

show endothelial function is supported by the findings that the dermal clones and DP primary cultures only formed tube-like structures on Matrigel when grown in endothelial media. Additionally, the functional activity was consistent with a decrease in the levels of α -SMA mRNA in DP cells and an increase in the levels of FLT1 mRNA expression. These observations from DP primary cultures were also confirmed using the dermal clones established by our group [6].

A recent study using skin reconstitution assays to investigate different populations of DP cells sorted on the basis of CD133 and SOX2 expression showed that CD133-negative cells did not contribute to hair follicle formation. CD133-positive and SOX2-positive cells were shown to have the strongest ability to form DP and non-DP structures in the dermis [42]. We found that DP and DS primary cultures, and DP and DS clones all express SOX2. DP primary cultures and DP9 clones express CD133, interestingly DP4 clones do not. DP4 cells exhibited a greater degree of endothelial potential compared to the DP9 cells and this might represent the different potentials of CD133-positive versus CD133-negative cells.

To examine if these cells are of neural crest origin, we used the WNT1cre/ROSA26R and WNT1cre/YFP mice. In the Matrigel assay, we found that cells of neural crest origin formed capillary-like structures and identified a population of cells (2%) that express PECAM and are of neural crest origin. Experiments with the WNT1cre mice were primarily carried out to determine if the endothelial expression and functionality observed in primary cultures were actually a result of contaminating endothelial cells. A previous study by [39] showed that endothelial cells in the craniofacial region of mice are not neural crest derived. However, the anagen DP is composed of neural crest-derived cells. Immunofluorescence experiments examining YFP (WNT1cre) and PECAM expression confirmed that DP was neural crest derived and the endothelial cells that compose the capillaries within the DP are not of neural crest origin. However, these experiments did identify a novel population of cells in the anagen follicle that reside within the DS at the base of the DP that coexpress endothelial markers and are of neural crest origin. In vivo, neural crest-derived cells that express endothelial markers exist in small numbers and reside in the vascular sinus surrounding the DS. These cells also express dermal markers, for example, α -SMA. A similar population of cells has been identified in the rat vibrissae follicles that express both vascular and dermal markers.

Nestin-expressing cells located within the bulge region of the follicle have been shown to form functional capillary networks in the dermis after transplantation of hair follicle [26], and comprise capillary networks after wound healing. These capillary networks have been shown to effectively integrate with the hosts dermal vascular network [43] and respond to angiogenic signals during tumor vascularization [44]. The proportion of nestin-expressing cells in functional blood vessels decreases with time after wound healing, suggesting that these cells act as a first response in capillary formation during wound healing [43]. It is possible that the population of neural crest-derived cells identified in this study may play a similar role: as a first response to wound healing.

Therefore, the findings of this study are 2-fold: first, that the plasticity of DP cells extends to being able to differentiate down an endothelial lineage. Primary cultures upregulate expression of FLT1 mRNA and can function similar to en-

dothelial cells in a range of in vitro assays. This ability appears to be associated with the whole population of DP cells, and we have confirmed that neural crest-derived DP cells have the ability to differentiate down an endothelial lineage. A second novel finding of this article is that in vivo a small population of neural crest-derived dermal cells expressing endothelial markers exist in the DS. It appears that functionally DP cells do not contribute significantly to the vasculature within the follicle. However, the small but consistent numbers of neural crest-derived cells expressing endothelial markers observed in this study suggest that these cells do have an important functional role in the skin.

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Author Disclosure Statement

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