Human dermal stem cells differentiate into functional epidermal melanocytes

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Summary
Melanocytes sustain a lifelong proliferative potential, but a stem cell reservoir in glabrous skin has not yet been found. Here, we show that multipotent dermal stem cells isolated from human foreskins lacking hair follicles are able to home to the epidermis to differentiate into melanocytes. These dermal stem cells, grown as three-dimensional spheres, displayed a capacity for self-renewal and expressed NGFRp75, nestin and OCT4, but not melanocyte markers. In addition, cells derived from single-cell clones were able to differentiate into multiple lineages including melanocytes. In a three-dimensional skin equivalent model, sphere-forming cells differentiated into HMB45-positive melanocytes, which migrated from the dermis to the epidermis and aligned singly among the basal layer keratinocytes in a similar fashion to pigmented melanocytes isolated from the epidermis. The dermal stem cells were negative for E-cadherin and N-cadherin, whereas they acquired E-cadherin expression and lost NGFRp75 expression upon contact with epidermal keratinocytes. These results demonstrate that stem cells in the dermis of human skin with neural-crest-like characteristics can become mature epidermal melanocytes. This finding could significantly change our understanding of the etiological factors in melanocyte transformation and pigmentation disorders; specifically, that early epigenetic or genetic alterations leading to transformation may take place in the dermis rather than in the epidermis.

Key words: 3D model, Melanocyte, Stem cells, Dermal reservoir

Introduction
In vertebrate development, melanocytes originate from the neural crest and undergo a complex process of fate specification, proliferation, survival and differentiation, before finally residing in the epidermis (Le Douarin, 1999). The presence of a melanocyte stem cell niche has been shown in hair follicles of mouse and human skin (Nishimura et al., 2002; Yu et al., 2006). However, no obvious spatially restricted niche of melanocyte stem cells has been found in glabrous areas, which are abundant in melanocytes. Melanocyte stem cells in hair follicles lose their self-renewal capacity with aging, leading to hair graying. By contrast, skin melanocytes sustain lifelong proliferative potential. Although it is a rare case, repopulation of melanocytes occurs in vitiliginous areas after UVA treatment, even in glabrous skin (Davids et al., 2009). These data suggest that extrafollicular melanocyte stem cells might exist, and could potentially function as a reservoir for melanocytes in postnatal epidermis.

Neural crest progenitors and stem cells have been characterized in recent years in murine, avian and human models. Using in vitro cultures of single neural crest cells or by labeling single neural crest cells with vital dyes, the neural crest cell population was found to contain pluripotent progenitors as well as early restricted precursors (Bronner-Fraser and Fraser, 1988; Morrison et al., 1999; Trentin et al., 2004). A pure or enriched population of neural crest stem cells has been isolated from mouse trunk neural tubes using low-affinity nerve growth factor receptor (NGFRp75) as a marker (Stemple and Anderson, 1992). These NGFRp75-positive cells have self-renewal capacity, as well as displaying multipotent differentiation properties, and might thus represent murine neural crest stem or progenitor cells. How neural crest cells become committed to the melanocytic lineage and what factors control the survival, proliferation, migration and differentiation of melanocyte precursors remains largely unknown. Such issues are of great importance for understanding the mechanisms of several pigment cell pathologies, including melanoma (Dupin and Le Douarin, 2003). In haired skin areas, increasing evidence suggests that NGFRp75-positive multipotent adult stem cells do exist within hair follicle dermal papillae and that they might contribute to the renewal of neural and non-neural cells, including melanocytes (Fernandes et al., 2004; Toma et al., 2001; Yu et al., 2006). These findings led us to hypothesize that the extrafollicular reservoir for melanocytes might be located in the dermis where stem cells would be exposed to less physiological or chemical stress than in the epidermis.

Here, we show that the dermis-derived stem cells (termed dermal stem cells or DSCs) from human glabrous skin are multipotent, similarly to neural crest stem cells, and can differentiate into melanocytes. Using a three-dimensional (3D) skin reconstruct model, we demonstrate for the first time that DSCs differentiating into melanocytes migrate from the dermis to the epidermis, suggesting the existence of a reservoir for melanocytes in the more protective dermal layer of the skin.

Results
Isolation of dermal spheres from neonatal human foreskins
Previous studies have shown that multipotent precursor cells that can be differentiated both into neural and mesodermal cell types are
present in human foreskins (Toma et al., 2005). With the aim of finding melanocyte precursors in locations other than hair follicles, the epidermis and basement membrane of foreskins were dissociated from the dermis. Dermis-derived single cells were then grown in HESCM4 medium, which is sufficient to maintain human embryonic stem cells in an undifferentiated state in the absence of feeder cells (Fig. 1A). Although most of the single cells gradually died, some cells started forming clusters within 5-7 days of culture (Fig. 1B). After 10-14 days, characteristic 3D spheres were observed (Fig. 1C), which were termed ‘dermal spheres’. The majority of dermal spheres adhered to plastic, and only a small number of spheres floated. The dermal spheres were easily detached by tapping the flasks as they separated from the monolayer cells, which were strongly attached to the culture flasks. The subcultured spheres showed the same morphology as those in the primary culture (Fig. 1D). Nineteen of 24 (79%) foreskin samples formed spheres, and the sphere-forming efficiency of single cells was approximately 0.085% (3840 spheres from 4.6×10⁶ single cells). To test whether other cell populations derived from foreskins form spheres in HESCM4 medium, we isolated melanocytes and fibroblasts from foreskins and cultured them in HESCM4 medium. After 1 week of culture, most melanocytes started to die, and all cells died within 2 weeks (Fig. 1E). Although fibroblasts readily grew in HESCM4 medium, they did not form spheres (Fig. 1F). These results indicate that the dermal sphere-forming cells are unique and are neither melanocytes nor fibroblasts.

**Dermal spheres express neural crest stem cell and ES cell markers**

To investigate the expression of stem cell markers in dermal spheres, they were cytopun onto slides and stained by immunocytochemistry. After testing more than 10 human ES and neural crest stem cell markers (data not shown), three markers (OCT4, nestin and NGFRp75) were found to be consistently expressed in most of the dermal spheres (Fig. 2A-C). The human ES cell marker OCT4 showed a characteristic punctate nuclear staining pattern both in dermal spheres and in H9-derived embryoid bodies (EBs) (Fig. 2A). The majority of cells within the dermal spheres were strongly positive for the neural crest cell markers nestin and NGFRp75 (Fig. 2B,C). NGFRp75 was expressed only in the dermal spheres, not in single cells surrounding the spheres. Additionally, not every cell in the spheres expressed NGFRp75, indicating that the spheres were composed of heterogeneous populations. Some of the NGFRp75⁺ cells coexpressed OCT4 (Fig. 2D). Immunochemistry revealed that OCT4 was typically localized to nuclei (stained blue with DAPI). Scale bars: 100 μm (for EB) and 70 μm (DSC). (B) Dermal spheres are positive for the neural crest stem cell marker nestin (green). Scale bar: 100 μm. (C) The neural crest stem cell marker NGFRp75 (green, cytoplasmic staining) is expressed in dermal spheres. Nuclei are stained blue with DAPI. Scale bar: 100 μm. (D) Upper panel, co-staining dermal spheres for OCT4 (green) and NGFRp75 (red) shows distinctive cells that coexpress both markers. Scale bar: 40 μm. Lower panel, control staining with mouse IgG and rabbit IgG. Scale bar: 100 μm. (E) FACS analysis of the cell surface marker NGFRp75 in a human foreskin dermal cell suspension. Fibroblasts (FF) and the WM3248 melanoma cell line were used as negative and positive controls, respectively.
However, the melanocytic markers HMB45 and tyrosinase-related protein 1 (TYRP1) were negative (data not shown), suggesting that mature melanocytes did not exist within the spheres. FACS analysis showed that 12% of the dermal cells were positive for NGFRp75 (Fig. 2E).

**Dermal spheres contain cells capable of self-renewal**

Self-renewal capacity is a common property of neural crest precursor cells (Trentin et al., 2004). We examined whether cells within the dermal spheres can undergo self-renewal by serial cloning in vitro. Three dermal sphere cell lines were cloned to test their self-renewing ability and to exclude contaminating cell populations. By 15 days, single cells derived from the spheres formed small colonies. A typical sphere appeared after approximately 90 days (Fig. 3A). We termed these cloned dermal sphere cells ‘dermal stem cells’ (DSCs). Some wells contained flattened cells that proliferated into monolayer cultures with a fibroblastic morphology, but they were unable to reform spheres (data not shown). Approximately 0.3% (3 out of 960) of the single dissociated spheres were capable of forming new spheres. The cells in reformed spheres were able to divide, similarly to EBs and human hair follicle stem cells (HFSCs), because proliferating cells were detected by Ki67 staining (Fig. 3B).

**DSCs differentiate into melanocytes and other neural crest-derived cell types**

The multipotency of DSCs was characterized by the induction of differentiation into several cell lineages. Re-formed spheres were enzymatically dissociated into single cells and were plated onto coated tissue-culture-grade plastic in differentiation medium. After 2-3 weeks under appropriate differentiation culture conditions, cells were characterized by immunostaining. When cultured under the same conditions that allowed the differentiation of human HFSCs into neurons (Yu et al., 2006), cells acquired a dendritic morphology and expressed β3-tubulin (Fig. 4A). They were also positive for neurofilament M (NFM) protein and MAP2 (not shown). Smooth muscle cells originate from the mesoderm layer and it was recently shown that they can be differentiated from human embryonic stem cells and mesenchymal stem cells (Gong et al., 2008; Lu et al., 2009). Our previous work showed that human HFSCs can differentiate into smooth muscle cells (Yu et al., 2006). In smooth

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**Fig. 3. DSCs are capable of self-renewal and proliferation.** (A) Limiting dilution assays showed that a single cell derived from a DSC sphere re-formed a sphere. A typical sphere appeared after approximately 90 days. (B) Ki67 staining detected proliferating cells in an embryoid body (EB), a DSC sphere and a human hair follicle stem cell sphere (HFSC). Nuclei are stained blue with DAPI; red phalloidin (Pha) staining allows visualization of actin filaments. Scale bars: 100 μm (A) and 50 μm (B).

**Fig. 4. Further characterization of the stem cell properties of DSCs by induction of differentiation into several cell lineages.** (A-D) DSC spheres were enzymatically dissociated into single cells and plated in tissue culture-grade chamber slides in differentiation medium. After 14 days, lineage-specific staining was performed. Scale bar: 50 μm. Neural differentiated cells become immunoreactive to β3 tubulin (A). Differentiated smooth muscle cells express smooth muscle actin (SMA) (B). Differentiated chondrocytes are positive for collagen II (C). Differentiated adipocytes are detected by Oil-red-O staining (D). (E-H) Melanocytic differentiation was performed in melanocyte differentiation medium. Immunofluorescent staining reveals that most differentiated cells are positive for the melanocyte markers MITF (E) DCT (F) and S100 (G), and the pigmentation marker HMB45 (H). Scale bar: 50 μm.
muscle differentiation medium, dermal-sphere-derived cells were successfully induced into smooth muscle cells that acquired abundant cytoplasm and displayed immunoreactivity for smooth muscle actin (SMA) (Fig. 4B). DSCs were also able to differentiate into chondrocytes expressing collagen II (Fig. 4C). Adipogenesis was seen when dermal sphere cells were cultured in adipocyte differentiation medium. After 2 weeks of culture, lipid droplets were present in some cells, which could be detected by Oil-red-O staining (Fig. 4D). Finally, DSCs were subjected to conditions favoring melanocyte formation as defined with human ES cells and HFSCs (Fang et al., 2006; Yu et al., 2006). After 2-3 weeks of culture in melanocyte differentiation medium, most of the cells had died and floated, whereas some of the attached cells had developed dendritic processes and expressed the melanocyte markers MITF, DCT, S100 and HMB45 (Fig. 4E-H). Moreover, a significant decrease in NGFRp75 expression was observed in induced melanocytes compared with dermal sphere cells (supplementary material Fig. S1).
DSCs migrate to the epidermis and differentiate into melanocytes of 3D skin reconstructs

Unlike epidermal melanocytes or melanocyte stem cells in the hair follicle bulge region, DSCs cultured in HESCM4 medium did not express melanocyte-lineage-specific markers such as TYRP1 and HMB45 (data not shown). Since melanocytes in mouse skin are localized in hair follicles only, suggesting that they have different regulatory properties to human epidermal melanocytes, mouse skin might not be a suitable model to study the mechanisms of human melanocyte differentiation. By contrast, the behavior of melanocytes in human skin reconstructs reflects the physiological situation in human skin more accurately (Haake and Scott, 1991; Meier et al., 2000). Thus we, used human 3D skin reconstructs to further evaluate the differentiation and migration of melanocytes from DSCs (Fig. 5A-C). Skin reconstructs consist of a ‘dermis’ of collagen with embedded fibroblasts and an ‘epidermis’ of multi-layered keratinocytes with melanocytes at the basement membrane separating the epidermis from the dermis. We first introduced melanocytes differentiated as a monoculture from DSCs into skin reconstructs to test whether they could function as bona fide epidermal melanocytes. When the skin reconstructs were harvested and sectioned, the DSC-derived melanocytes were localized at the basement membrane zone of the skin reconstructs in the same manner as epidermal melanocytes in human skin. They were pigmented, as detected by Fontana-Masson staining, and expressed the melanocyte markers S100 and TYR (Fig. 5A). We further questioned whether we could directly induce melanocyte differentiation and migration from DSCs in the 3D context. First, DSCs were embedded in collagen I, which constitutes a large part of the dermis of skin reconstructs. After 2-3 days, we observed migratory ability of single cells of the spheres in collagen I (Fig. 5B). Next, we embedded NGFRp75-positive DSCs together with human foreskin fibroblasts into the dermal layer of skin reconstructs and seeded keratinocytes on top of the dermis. After two weeks of culture, NGFRp75-positive cells coexpressed the melanocytic marker HMB45 (Fig. 5C). Strikingly, some HMB45-positive cells were now seen in the epidermis where melanocytes normally reside. Melanocytes found in the basal layer of the epidermis no longer expressed NGFRp75, suggesting that it had been downregulated by keratinocytes (Fig. 5C). To track long-term cell migration, DSCs were transduced with a lentiviral vector encoding GFP and then incorporated into the dermis of skin reconstructs (Fig. 5D). At day 5 after seeding keratinocytes, single cells started migrating out from spheres. At day 8, few cells reached the epidermis-dermis interface. At day 10, GFP-positive cells were tightly aligned at the basement membrane position, and the number of these cells dramatically increased at day 12 (supplementary material Fig. S2).

DSC-derived melanocytes gain E-cadherin expression upon contact with keratinocytes.

The control of cadherin expression is essential for neural crest migration and melanocyte localization (Nakagawa and Takeichi, 1998; Nishimura et al., 1999). Thus, we characterized the profile of cadherin expression during DSC differentiation to melanocytes. Both E-cadherin and N-cadherin were negative in dermal spheres before embedding into the dermis of the skin reconstructs (Fig. 6A,B). These data confirm that epidermal melanocytes, which express E-cadherin (Tang et al., 1994), are not contaminating in dermal spheres. After two weeks of culture in the skin reconstructs, all dermal cells including NGFRp75-positive cells were negative for both cadherins (Fig. 6C). A few cells residing in the dermis expressed N-cadherin; however, they did not express either NGFRp75 (Fig. 6D) or the melanocytic marker S100 (data not shown), suggesting that these cells are probably fibroblasts co-embedded with dermal spheres. S100-positive melanocytes that homed to the epidermis were all positive for E-cadherin and remained negative for N-cadherin (Fig. 6E,F).

NGFRp75-positive OCT4-positive cells are located in foreskin dermis

To determine the location of DSCs in situ, we analyzed protein expression of OCT4 and NGFRp75 in human foreskins. Highly OCT4-immunoreactive cells were found in the middle and lower dermis (Fig. 7A). These cells were small and round. A larger number of NGFRp75-immunoreactive cells were seen located in the entire dermal compartment (Fig. 7B). Most of the NGFRp75-positive cells were spindle shaped, which corresponds to the morphology of nerve fibers (Fig. 7B, arrows); however, some small and round NGFRp75-positive cells were also seen as single cells (Fig. 7B, arrowheads). Furthermore, double staining with OCT4 and NGFRp75-antibodies demonstrated the presence of a small population that co-expressed both antigens (Fig. 7C). Those cells were round and had small amounts of cytoplasm. NGFRp75 was strongly expressed at the peripheral part of the cells and punctate OCT4 staining was seen in nuclei.

![Fig. 6. DSC-derived melanocytes gain E-cadherin expression upon contact with keratinocytes.](image-url)
Discussion

In the present study, we demonstrate that dermal stem cells isolated from human foreskin are capable of self-renewal and differentiation into multiple lineages. Using a 3D skin-equivalent model, we show that the dermal stem cells can differentiate into HMB45-positive melanocytes inside the dermis. These dermal melanocytes are able to migrate to the basal layer of the epidermis to become functionally more mature, E-cadherin-positive epidermal melanocytes upon contact with the surrounding keratinocytes.

Nishimura and co-workers (Nishimura et al., 2002) reported that postnatal stem cells for melanocytes found in hair follicles express Dct and appear to be restricted to the melanocyte lineage. Unlike these stem cells from mouse trunk hair follicles, human DSCs do not express any known melanocyte markers. Similarly to neural crest stem cells from other embryonic and postnatal sources, the DSCs expressing NGFRp75, OCT4 and nestin are multipotent and are able to generate neuronal and non-neuronal lineages. DSCs can generate melanocytes under the same conditions as human embryonic stem cells (Fang et al., 2006).

The existence of multipotent skin-derived precursor cells (SKPs) has been shown in human foreskins (Toma et al., 2005). However our DSCs appear to be different from SKPs growing in suspension and differentiating into neural lineages but apparently not melanocytes. DSCs shared mesenchymal markers such as slug and snail (data not shown), which are expressed in SKPs. However, the neural crest marker NGFRp75 was expressed only at low or undetectable levels in SKPs, whereas it is highly expressed in DSCs. Additionally, some of the DSCs express an embryonic stem cell marker, OCT4. Collectively, these data suggest that DSCs are similar to, but distinct from SKPs. Unlike the previous studies using medium containing bFGF and EGF, defined to grow neural stem cells, we used human embryonic-stem-cell-based medium conditioned with mouse embryonic fibroblasts. This culture medium maintained the ability of DSCs to differentiate into not only neural and skeletal derivatives but also melanocytes. Our data suggest that factors derived from fibroblasts support the maintenance of DSCs in a more immature state.

It is largely unknown how multipotent DSCs exposed to several signals in vivo integrate them to regulate the outcome of a particular phenotype; however, in vitro studies indicate that the integration is regulated by stem-cell-intrinsic differences in the relative sensitivity and timing of responses to growth factors (Shah and Anderson, 1997). Several growth factors have been identified that direct melanocyte differentiation. It was shown that a synergistic interplay of three growth factors – EDN3, SCF and WNT3a – is required for the differentiation of ES cells into melanocytes. Our previous study suggested that these three factors perform subtle but differing roles in human ESC-to-melanocyte differentiation (Fang et al., 2006). During embryonic development, EDN3 has several roles in regulating the melanocyte lineage, supporting the migration, survival, proliferation and differentiation of melanoblasts (Dupin and Le Douarin, 2003). Knockout mice for either Edn3 or the endothelin-B receptor exhibit a nearly complete loss of melanocytes (Baynash et al., 1994), suggesting that this pathway is crucial in the development of melanocyte populations. Another factor that participates in melanocyte differentiation is SCF, the ligand of c-KIT. Recently, Motohashi and colleagues showed that c-KIT-positive melanoblasts (non-pigmented, Tytp1-positive and nestin-negative cells) from murine skin retain the potential to differentiate into other neural crest cell derivatives, such as neurons, glial cells and smooth muscle cells (Motohashi et al., 2009). Their study indicates that c-KIT-SCF signaling has a crucial role in supporting the survival and multipotency of neural crest stem cells. However, it has not yet been addressed whether this is also the case in human skin. The last factor – Wnt – has a variety of roles in neural crest development, including the induction and proliferation of the melanocyte lineage (Le Douarin, 1999). The absence of Wnt ligands and of b-catenin from neural crest cells leads to the loss of expression of MITF (microphthalmia-associated transcription factor) and melanocyte differentiation markers (Dorsky et al., 1998; Hari et al., 2002).
Neuronal and glial fates are induced by high levels of Wnt signaling, thus it is likely that Wnt3a signaling determines the melanocyte fate of neural crest cells. Interestingly, DSCs differentiate into melanocytes without exogenous Wnt3a when incorporated into the dermis of skin reconstructions. It is likely that when unidentified cells are embedded into the skin reconstructions, they first interact with fibroblasts and later are attracted by keratinocytes inducing differentiation and migration. It was shown that Wnt3a was expressed at the basal layer of the human epidermis (Jia et al., 2008), suggesting that keratinocytes might have a role in regulating the melanogenic commitment of DSCs in the skin.

It is still not clear whether DSCs differentiate to melanocytes first, then migrate to the epidermis or if they migrate first and then complete their differentiation to melanocytes. Our study shows that DSC-derived melanocytes coexpress both NGFRp75 and the melanocytic marker HMB45 when they stay in the dermis, whereas they lose expression of the former once they anchor to the basement membrane. The data support the idea that completion of melanocyte differentiation is determined by physical interactions with keratinocytes. Because NGFRp75 influences a variety of cellular functions depending on the cellular context (Cragonolini and Friedman, 2008), it is not surprising that this receptor might have a physiological role in DSCs. In fact, we observed that inhibiting NGFRp75 by infecting dermal cells with a lentiviral shRNA vector abolished the formation of spheres (unpublished results), which suggests that NGFRp75 is crucial for the self-renewal capacity of DSCs.

Classical cadherins, such as E-cadherin, N-cadherin and P-cadherin, determine melanocyte positioning in the skin. Specifically, the expression of E-cadherin has a key role in guiding melanocyte progenitors to the epidermis (Nishimura et al., 1999). In mice, most melanocytes eventually disappear from the epidermis during postnatal life (Hirobe, 1984), whereas in human skin, melanocytes remain in the epidermis throughout the entire life span. The growth of epidermal melanocytes is tightly regulated by adjacent keratinocytes via homophilic interactions of E-cadherin. An escape from the control of keratinocytes as a consequence of the loss of E-cadherin is one of the critical steps of malignant transformation of melanocytes. Interestingly, DSCs were positive for neither E- nor N-cadherin. It is conceivable that the lack of cadherins allows melanocyte precursors to migrate to the epidermis. Mouse melanocyte progenitors do not express E-cadherin in the early stages and upregulate the E-cadherin expression level markedly before emigrating into the epidermis. We did not observe any E-cadherin-positive cells in the dermis of skin reconstructions, suggesting that DSC-derived melanocytes acquire E-cadherin expression only upon contact with keratinocytes.

In summary, we isolated multipotent DSCs from human foreskin dermis. These cells are capable of forming spheres, they express the neural crest marker NGFRp75 and the undifferentiated marker OCT4, display an extensive self-renewal capacity and can successfully differentiate into several derivatives of neural crest, including melanocytes. Cultures of 3D skin reconstructions revealed that intradermal DSCs can differentiate into melanocytes, which can then migrate to the basal epidermis and establish bona fide communication with keratinocytes through upregulation of E-cadherin. These data strongly indicate that stem cells in the dermis constitute a reservoir for epidermal melanocytes. Thus, our work provides a window of opportunity to explore the possibility of DSC involvement in physiological and pathological events such as tissue repair and neoplastic changes of melanocytes.

Materials and Methods

Cell culture

DSCs were isolated from human foreskins. Briefly, foreskins derived from circumcisions of newborns were washed with Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). Excess adipose tissues were removed, and the skin specimens were cut into approximately 0.5-0.5 cm² pieces and were incubated in 0.5% trypsin (Difco) II (Invitrogen) at 4°C. After 18 hours, the epidermis was manually removed from the dermis. The basement membrane attached to the dermis was carefully scraped away using a surgical blade to remove all melanocytes. The dermis was minced into 1-2 mm squares and incubated in 1 mg/ml collagenase type IV (Invitrogen) for 24 hours at room temperature. The suspensions were diluted in 10% DMEM/F12 medium and were serially filtered through 100 μm, 70 μm and 40 μm cell strainers. Cells were cultured in HESCM4 medium, which contains 80% KnockOutDMEM/F12 medium (Invitrogen), 20% KnockOutDMEM/F12 medium (Invitrogen), 200 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 1% nonessential amino acids (Invitrogen) and 4 ng/ml basic fibroblast growth factor (bFGF) (Gibco). The medium was conditioned by using it overnight as a growth medium for mouse embryonic fibroblasts (MEFs) as described previously (Xu et al., 2001). The conditioned medium was mixed with fresh non-conditioned medium at a 1:3 ratio, sterilized by filtration, and supplemented with additional bFGF at 4 ng/ml before use. To subculture cells, the detached spheres were incubated in 0.25% trypsin with EDTA and 1 mg/ml collagenase IV for 5 minutes at 37°C and split at 1:3 ratios. The human embryonic stem cell line h9 was obtained from the WiCell Research Institute (Madison, WI) and cells were cultured on mitotically inactivated MEF feeder layers in HESCM4 medium. Human hair follicle stem cells (HFSs) were cultured in HESCM4 medium as described previously (Yu et al., 2006). WM3248 melanoma cells were cultured in Tu 2% medium which including 80% MCDHB153 (Sigma), 20% Leibovitz’s L15 (Invitrogen), 2% fetal bovine serum (FBS), 5 μg/ml insulin (Sigma) and 1.68 mM CaCl2 (Sigma). Human melanocytes and fibroblasts were isolated from foreskins and cultured in 254CF (Invitrogen) and DMEM with 10% FBS, respectively.

Flow cytometry

Single cells obtained from fresh human foreskin tissues were serially filtered with 100 μm, 70 μm, and 40 μm cell strainers. Cells were incubated with NGFRp75 FITC-conjugated antibodies (CD278-ITC) at 4°C. After 18 hours, the cells were washed with fresh non-conditioned medium at 4°C for 60 minutes at 4°C on a shaker. Mouse IgG was used as an isotype control. Propidium iodide staining was performed to exclude the dead cell population in FACS analyses. Cells were analyzed using a FACScan (Becton Dickinson) and FlowJo software (Tree Star, Ashland, OR).

Clonal analysis

To obtain single cells, dermal spheres were dissociated by incubation in 0.25% trypsin-EDTA, 1 mg/ml collagenase type 1a (Sigma), 1 mg/ml collagenase IV and 2% (w/v) bovine serum albumin (BSA) (Sigma) for 5 minutes at 4°C. Single cells were then cultured in HESCM4 medium and seeded in 96-well plates at 0.75 cell/well. After 24 hours, each well was scored for the presence or absence of a single cell. To each well containing a single cell, an additional 20 μl fresh HESCM4 medium was added every 3 days.

Differentiation assays

Dermal spheres were dissociated into single cells, as described above, before plating onto tissue culture-grade plastic-coated chamber slides (Fisher Scientific, Pittsburgh, PA). For melanocyte, chondrocyte and adipocyte differentiation, slides were coated with 10 ng/ml fibronectin (Becton Dickinson). For neuronal and smooth muscle cell differentiation, slides were coated with 0.1% Matrigel® (Becton Dickinson). Melanocyte differentiation medium contains 0.05 μM dexamethasone (Sigma), 1 μM insulin-transferin-selenium (ITS) (Sigma), 1 mg/ml linoleic acid-bovine serum albumin (LA-BSA) (Sigma), 30% low-glucose DMEM (Invitrogen), 20% MCDHB201 (Sigma), 10-4 M L-ascorbic acid (Sigma), conditioned medium of mouse L-Wnt3a cells (American Type Culture Collection, Manassas, VA) (50%), 100 ng/ml stem cell factor (SCF) (R&D Systems, Minneapolis, MN), 100 mM endothelin-3 (EDN3) (American Peptide, Sunnyvale, CA), 20 pM cholera toxin (Sigma), 50 nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate (Sigma) and 4 ng/ml bFGF (R&D Systems).

Neuronal differentiation was performed as previously described (Fernandes et al., 2004). Single cells from spheres were plated in chamber slides coated with Matrigel® in DMEM/F12 (3:1) supplemented with 40 ng/ml bFGF and 10% FBS and were then incubated for 7 days. Cells were then cultured for an additional 7 days. Single cells were then cultured in the same medium without bFGF but with the addition of 10 ng/ml nerve growth factor, 10 ng/ml brain-derived neurotrophic factor (Peprotech, Rocky Hill, NJ) and 10 ng/ml NT3 (Peprotech).

For adipocyte differentiation, cells were cultured in medium containing low-glucose DMEM, 1% ITS (Sigma), 1 mg/ml LA-BSA (Sigma), 1 μM hydrocortisone (Sigma), 50 μM indomethacin (Sigma), 0.5 mM isobutylmethylxanthine (Sigma) and 10% horse serum (Invitrogen). For chondrocyte differentiation, cells were cultured in medium containing 90% high-glucose DMEM, 10% FBS, 1% ITS, 1 mg/ml LA-BSA, 50 mM dexamethasone and 60 μM transforming growth factor β1 (TGF-β1).
Isotype-matched IgG was used as a control. Primary antibodies were incubated with tubulin (TuJ1) (Millipore), collagen II (Millipore) and smooth muscle actin (Sigma).

Recombinant lentiviruses

Recombinant lentivirus vectors were produced to deliver CCR5-shRNA in vitro. The lentivirus vector was produced by co-transfection of human embryonic kidney 293T cells with four plasmids: a packaging defective helper construct, a Rev plasmid, a plasmid coding for a heterologous envelope protein and the H1U-1 vector.

Human skin reconstitutions

Skin reconstitutions were generated as described previously (Fukunaga-Kalabis et al., 2006). Briefly, inserts of tissue culture trays (Organogenesis, Canton, MA) were coated with 1 ml bovine collagen I (Organogenesis) and layered with 3 ml of 2% dialyzed fetal calf serum (Invitrogen), 4.5 ng/ml bFGF, 100 nM EDN3, and 10 ng/ml SCF. Cultures were kept submerged in medium containing 1 ng/ml epidermal growth factor (EGF) (Invitrogen) for 2 days, 0.2 mg/ml EGF for another 2 days, then raised to the air–liquid interface via feeding from below with high-calcium (2.4 mM) medium. Two weeks later, skin reconstitutions were harvested, fixed in 10% neutral buffered formalin for 3 hours, and processed by routine histological methods.

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