Histochem Cell Biol (2010) 133:455–465
DOI 10.1007/s00418-010-0684-z

ORIGINAL PAPER

Differential expression of stem cell markers in human follicular bulge and interfollicular epidermal compartments

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Accepted: 23 February 2010 / Published online: 13 March 2010

Abstract Although skin contains a number of stem cell repositories, their characterization has been hindered by a lack of specific markers and an unclear in vivo localization. In this study, we whole mounted single human scalp hair follicles and examined their profiles using in situ immunohistochemistry and multicolor immunofluorescence in search of markers to distinguish between stem cells residing in the interfollicular epidermis (IFE) and bulge. Our study revealed that expression of several biomarkers localized uniquely to the basal IFE (CD34 and CD117), bulge region (CD200), or both (CK15, CD49f, and CD29). In addition, we found that both basal IFE and bulge stem cells did not express CD71 or CD24 suggesting their potential utility as negative selection markers. Dermal papilla but not basal IFE or bulge stem cells expressed CD90, making it a potential positive selection marker for dermal hair follicle stem cells. The markers tested in this study may enable pursuit of cell sorting and purification strategies aimed at determining each stem cell population's unique molecular signature.

Keywords Epidermis · Hair follicle · Stem cell · Cell surface marker

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Abbreviations

DP	Dermal papilla
HF	Hair follicle
IFE	Interfollicular epidermis
IRS	Inner root sheath
ORS	Outer root sheath
SG	Sebaceous glands

Introduction

The epidermis forms the outer protective barrier of the body and regenerates throughout life. Similar to the epidermis, hair follicles (HFs) are self-renewing structures but differ via their distinct proliferative cycle consisting of three phases: growth (anagen), involution (catagen), and quiescence (telogen). Regeneration of the epidermis and HF involves several unique epithelial stem cell populations, some of which also participate in the repair of the skin after injury (Abbas and Mahalingam 2009). Although stem cells have the capacity to differentiate into all epidermal lineages in the skin, evidence suggests that each is derived from a unique stem cell compartment (Ghazizadeh and Taichman 2001; Ito et al. 2005; Levy et al. 2005, 2007; Owens and Watt 2003). HF regeneration also involves stem cell populations that reside in the dermal sheath and papilla (DP) (Jahoda et al. 2003; Waters et al. 2007).

In human skin, the long-lived, slow-cycling, and high proliferative potential stem cell compartment within the interfollicular epidermis (IFE) is located in the basal layer (Ghazizadeh and Taichman 2005; Lavker and Sun 1982, 2000). In the HF, stem cells reside in a discrete microenvironment called the bulge, which extends from the insertion point of the sebaceous gland duct to the attachment site for the arrector pili muscle (Montagna 1962). During each hair

cycle, quiescent bulge stem cells are stimulated to exit the stem cell niche, proliferate, and differentiate to regenerate the HF (Blanpain et al. 2004; Morris et al. 2004; Oshima et al. 2001; Taylor et al. 2000; Tumbar et al. 2004). In addition, the bulge stem cells have been shown to differentiate into epithelial, mesenchymal, and neural cells (Janes et al. 2002; Yu et al. 2006). Injury to the epidermis results in migration of the bulge cells to the epidermis, where they then contribute to wound repair and reepithelization (Ito et al. 2005; Levy et al. 2005). However, more recent evidence indicates that in normal states, bulge stem cells do not contribute to IFE reconstitution (Cotsarelis 2006; Ito et al. 2005).

There remains considerable debate about which markers are best suited to identify bulge stem cells (Cotsarelis et al. 1999). This is, in part, due to the fact that the human adult anagen bulge lacks unique morphological features (Akiyama et al. 1995; Lyle et al. 1998) and is continuous with the basal layer of both the IFE and SG (Stenn and Cotsarelis 2005). In the current study, we attempted to identify markers capable of distinguishing basal IFE and bulge stem cells using immunohistochemical localization and multicolor immunofluorescence of whole mounted single HFs derived from human scalp skin.

Materials and methods

Tissue samples

Excess skin from facelift procedures was collected with Stanford University IRB approval. Normal human scalp samples were obtained from five different donors. Skin samples were cut into 2 × 1 cm pieces and 31 single HFs were individually microdissected. All specimens were either fixed overnight in 4% neutral-buffered paraformaldehyde or frozen unfixed in OCT compound (Miles, Elkart, Indiana) on a frozen isopentane surface (cooled with liquid nitrogen). Paraformaldehyde-fixed tissue and HFs were transferred to 70% ethanol, and then embedded in paraffin. Both paraffin and frozen blocks were sectioned at 5 μm.

Antibodies

CD24, CD29, CD34, CD44, CD56, CD90, and CD133 were purchased from BD Biosciences-Pharmingen (San Jose, CA), CD49f and CD200 from AbD Serotec (Raliegh, NC), CK15 (C8/144B mAb) from DakoCytomation (Carpinteria, CA), and CD71 from BioLegend (San Diego, CA).

Immunohistochemistry

Paraffin-waxed sections were deparaffinized, rehydrated, and then subjected to antigen retrieval by steam heating for 15 min in pH 10 citrate buffer (Dako) at 85°C. Tissues were incubated with either various primary antibodies or rat IgG isotype control (BD Pharmingen) at the desired dilution (1:50–1:200) overnight at 4°C. Frozen sections were defrosted, air dried for 3 h, and fixed with acetone for 10 min at −20°C. They were then incubated with primary antibodies (diluted 1:50–1:200) overnight at 4°C. Signal was detected using the Vectastain Elite Mouse/Rat/Goat IgG detection kit (Vector Laboratories, Burlingame, CA), following the manufacturer's protocol, with diaminobenzidine (Dako) as the chromagen. Tissues were counterstained in Harris hematoxylin (Sigma-Aldrich, St. Louis, MO).

Double immunofluorescence

Frozen sections were defrosted, air dried, and fixed as above. Sections were blocked with 10% goat serum at room temperature for 10 min, and then incubated with the first primary antibody (mouse anti-CD200 IgG and mouse anti-CK15 IgG, diluted 1:50) at room temperature for 1 h. After three consecutive washes, they were incubated with goat anti-mouse IgG conjugated with rhodamine (Abcam, Cambridge, MA). Slides were washed and processed for a second round of blocking with 10% normal mouse serum at room temperature for 30 min. They were then incubated with the second primary antibody (mouse anti-CD24 IgG, mouse anti-CD29 IgG and mouse anti-CD34 IgG, diluted 1:50) at room temperature for 1 h. Slides were washed three times, and then incubated with goat anti-mouse IgG conjugated with FITC (Abcam) for 30 min. Slides were then washed, counterstained, and treated with mounting medium containing DAPI (Vector Laboratories).

Results

Microdissection and longitudinal mounting of single hair follicles

Human HFs are typically sectioned vertically due to difficulty in achieving full-length continuous longitudinal sections. However, vertical sections are suboptimal for immunolocalization studies. In order to overcome this limitation, we developed a novel method to whole mount single HFs after individually microdissecting them from human scalp skin under bright-field microscopy. This method dramatically increased the rate of obtaining full-length HF mounts.

Basal IFE and bulge stem cells express CK15, CD29, and CD49f

Cytokeratin 15 (CK15) is a marker of undifferentiated multipotent stem cells with high proliferative capacity (Jih

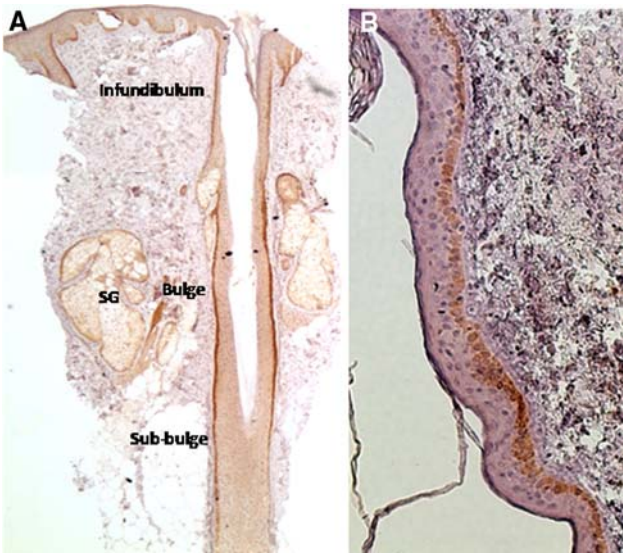

Fig. 1 Longitudinal section of a human scalp terminal anagen HF (**a**) and vertical section of human scalp epidermis (**b**) stained with anti-CK15 antibody (C8/144B). Immunoreactivity was visualized with diaminobenzidine (*brown* or *purple* color) and nuclei were counter-stained with hematoxylin. ×100 (**a**) or ×200 (**b**)

et al. 1999; Kanitakis et al. 1999). We used C8/144B, a monoclonal antibody originally raised against the carboxy-terminal peptide of the T cell protein CD8 (Mason et al. 1992), to detect the expression of CK15 in the IFE and HF. Immunohistochemistry showed strong CK15 staining in the outermost layer of the bulge ORS, the proximal isthmus, infundibulum, and outermost layer of the sub-bulge ORS (Fig. 1a). Homogeneous staining was also found in the basal IFE (Fig. 1a, b). Immunofluorescence studies confirmed that CK15 immunoreactivity was present in the outermost layer of the bulge ORS (Fig. 2a, b), but absent in the sub-bulge (Fig. 2c, d).

Previous studies have found that CD29 (β1 integrin) regulates the differentiation and proliferation of keratinocytes (Brakebusch et al. 2000; Zhu et al. 1999). In addition, CD49f (α6 integrin) bright keratinocytes have been identified as possible stem cells (Li et al. 1998). CD29 (Figs. 3a, 4a) and CD49f (Fig. 5c) expression was confined to the basal IFE. Homogeneous CD29 staining was present in the outermost layer of the ORS and the basement membrane throughout the entire HF length (Fig. 3b, c). Figure 5a, b shows that CD49f immunoreactivity is most intense in the outermost layer of the sub-bulge ORS. Positive staining extended to the bulb basement membrane and suprabasal sub-bulge ORS. Weak staining was observed in the outermost layer of the bulge ORS, but none in the infundibulum and arrector pili region. The hair matrix was positive for CD29 (Fig. 3b) and CD49f (Fig. 5a).

CD200 is expressed in the bulge but not the basal IFE

CD200 is a type I transmembrane glycoprotein previously used to enrich bulge stem cells from human HF suspensions (Ohyama et al. 2006). We found prominent CD200 staining in the outermost layer of the bulge ORS, infundibulum, and sebaceous gland (SG) duct (Figs. 4c, 6a). Minimal CD200 staining was found in the outermost layer of the sub-bulge ORS (Figs. 4b, 6a). Unlike CK15, no positive staining of CD200 was detected in the entire IFE (Figs. 4a, 6b).

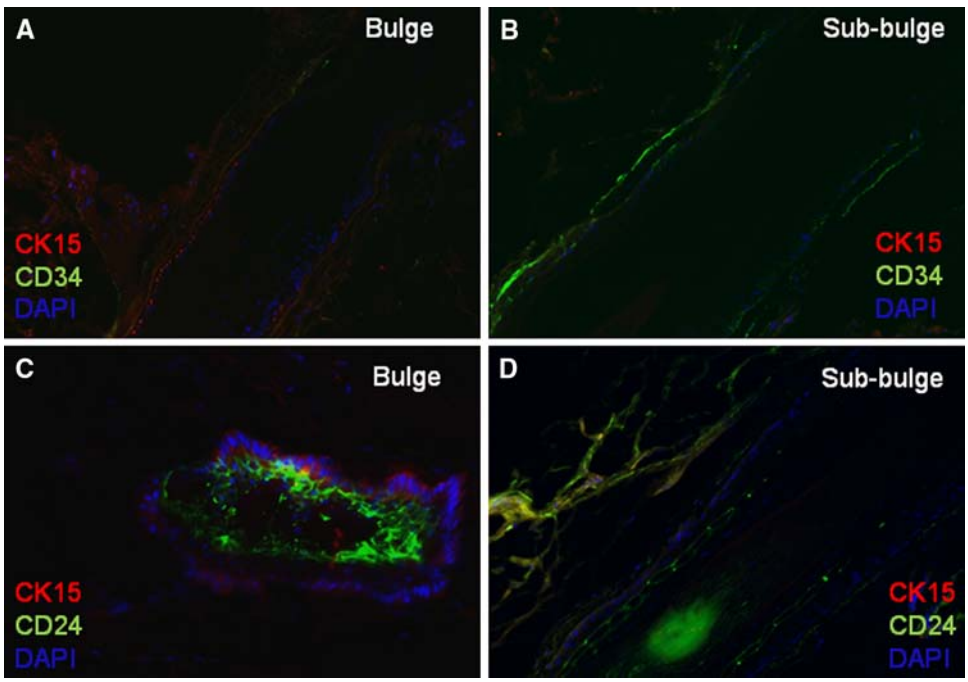

Fig. 2 Multicolor immunofluorescence of a vertically sectioned human scalp anagen HF stained for CK15 (**a**–**d**), CD24 (**c**, **d**), or CD34 (**a**, **b**) in either the bulge (**a**, **c**) or sub-bulge (**b**, **d**) region (×400)

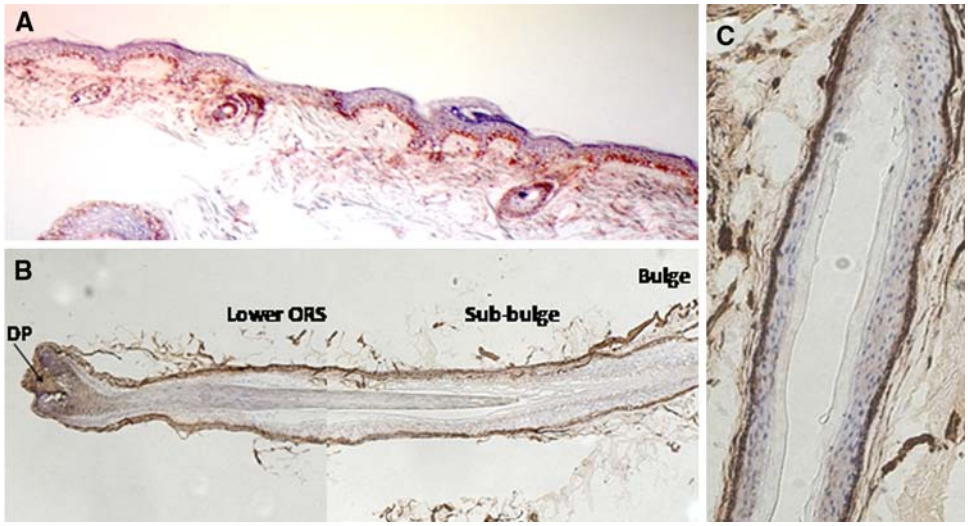

Fig. 3 Representative vertical section of human scalp epidermis (**a**) or longitudinal section of a human scalp HF (**b**, **c**) immunostained for CD29. ×100 (**a**, **b**) or ×200 (**c**)

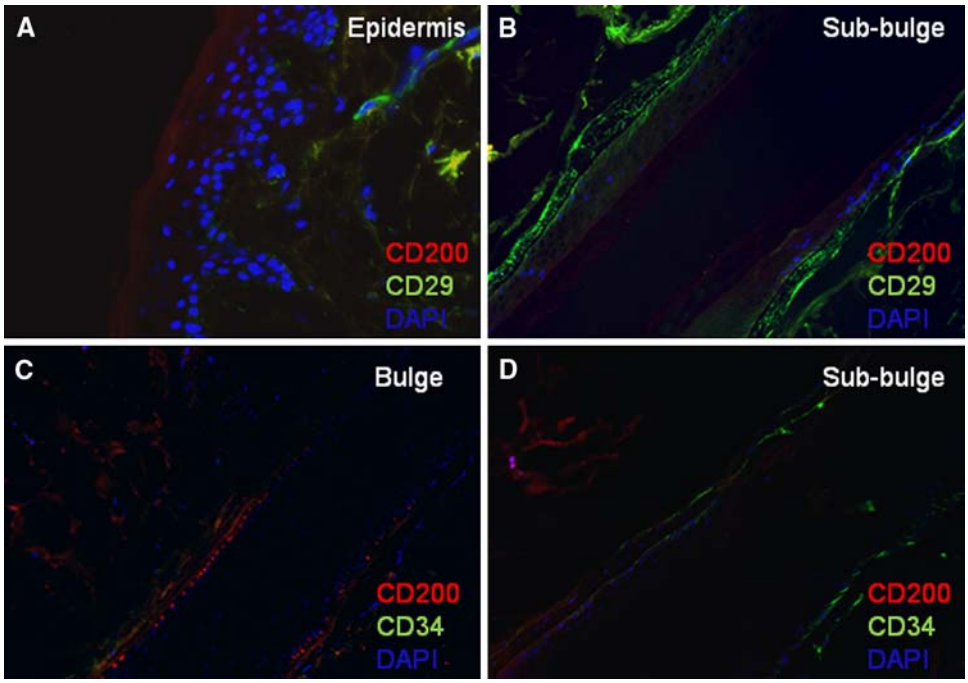

Fig. 4 Multicolor immunofluorescence of a vertically sectioned human scalp anagen HF stained for CD200 (**a**–**d**), CD29 (**c**, **d**), or CD34 (**c**, **d**) in either the epidermis (**a**), sub-bulge (**b**, **d**), or bulge (**c**) (×400)

Multicolor immunofluorescence revealed that CD29 positive basal IFE and sub-bulge ORS cells were negative for CD200 (Fig. 4a, b).

CD34 and CD117 are expressed in the basal IFE but not the bulge

CD34 is a 105–120 kDa, heavily glycosylated, transmembrane protein expressed on early hematopoietic stem and progenitor cells (Krause et al. 1994). Figure 7a illustrates that CD34 was not detected in the bulge, although positive staining was observed in the outermost layer of sub-bulge ORS and lower ORS. CD34 positive basal ORS cells rarely co-stained for CD200 or CK15 in the sub-bulge (Figs. 2b, 4d). Similarly, CD200 and CK15 positive bulge basal ORS cells did not stain positively for CD34 (Figs. 2a, 4c). Staining of vertical sections illustrated that CD34 was expressed in the basal IFE (Fig. 7b).

CD117 signaling is important for melanoblast and/or melanocyte migration, proliferation, and differentiation during embryogenesis, as well as for maintaining postnatal cutaneous melanogenesis (Besmer et al. 1993; Botchkareva et al. 2001; Hachiya et al. 2004). However, its role in epidermal and hair follicle regeneration remains unknown. We found CD117 staining in the outermost layer of the infundibulum and in the hair matrix (Fig. 8a, b). In the IFE, CD117 staining was restricted to the rete ridge portion (Fig. 8c).

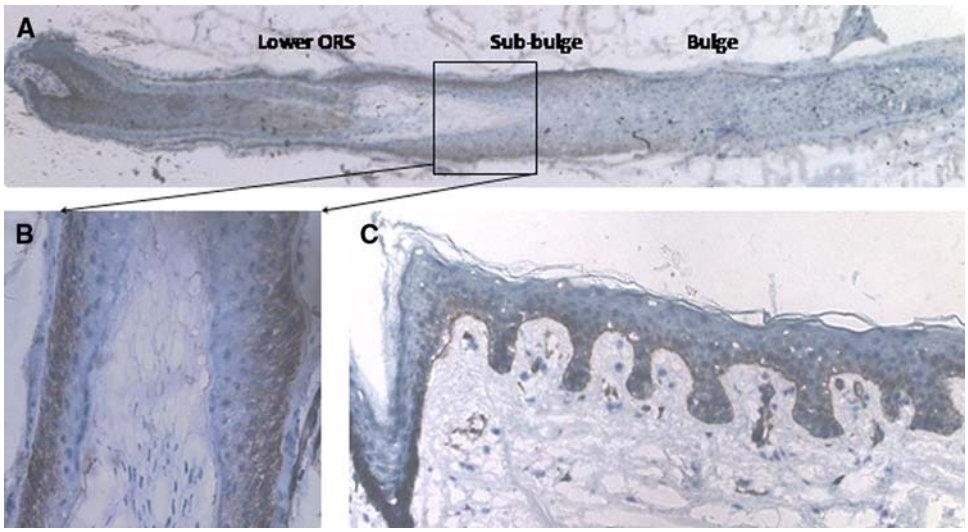

Fig. 5 CD49f immunoreactivity in a longitudinally sectioned human HF (**a**, **b**) or vertically sectioned epidermis (**c**). ×100 (**a**) or ×200 (**b**, **c**)

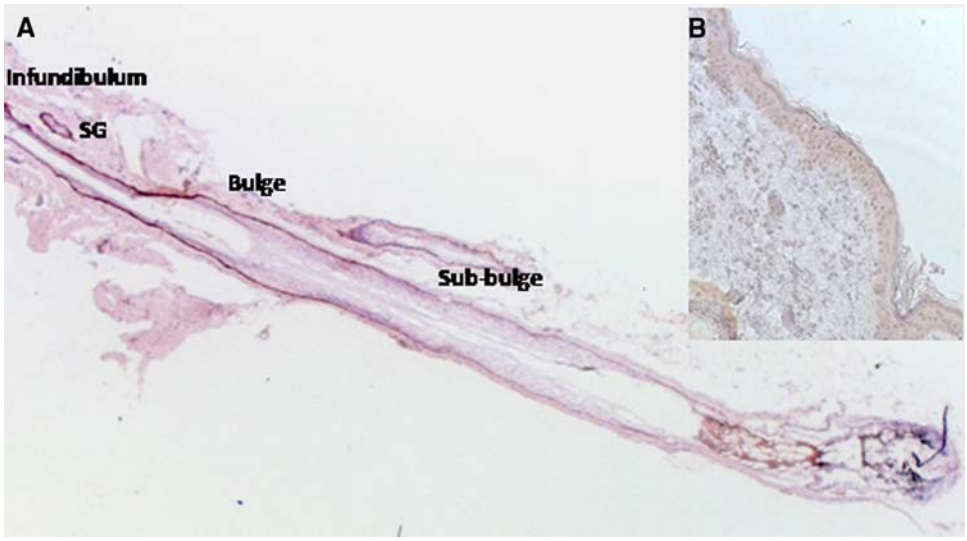

Fig. 6 CD200 immunoreactivity in a longitudinally sectioned human scalp HF (**a**) or vertically sectioned human scalp epidermis (**b**). ×100 (**a**) or ×200 (**b**) (*SG* sebaceous gland)

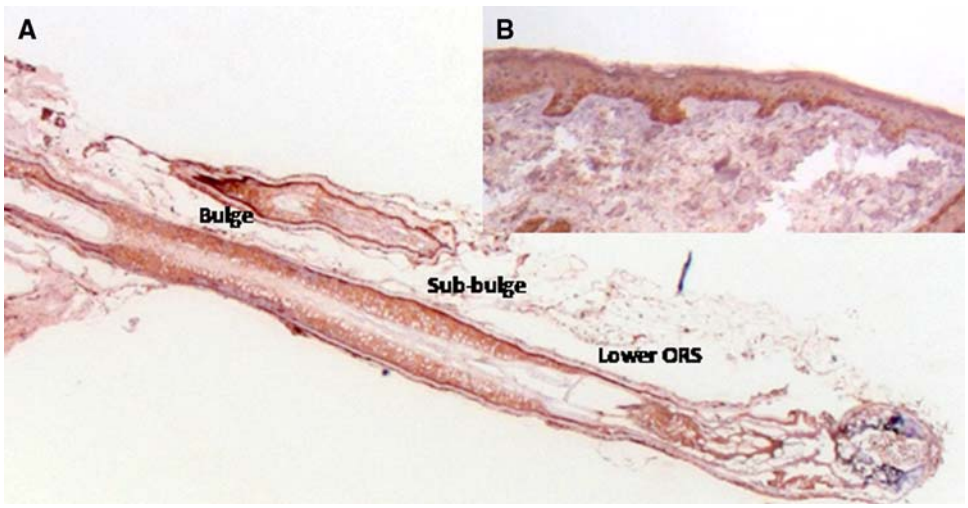

Fig. 7 CD34 immunoreactivity in a longitudinally sectioned human scalp HF (**a**) or vertically sectioned human scalp epidermis (**b**). ×100 (**a**) or ×200 (**b**)

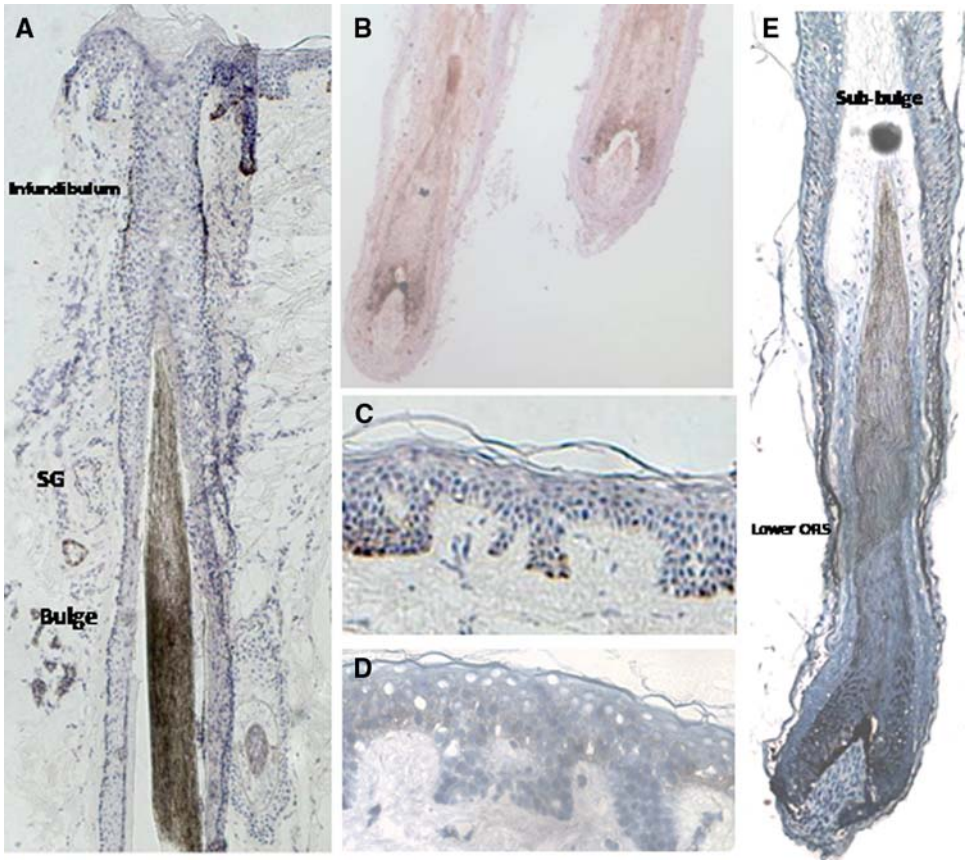

Fig. 8 CD117 (**a**–**c**) or CD71 (**d**–**e**) immunoreactivity in a longitudinally sectioned human scalp HF (**a**, **b**, **e**) or vertically sectioned human scalp epidermis (**c**, **d**). ×100 (**a**–**c**) or ×200 (**d**, **e**)

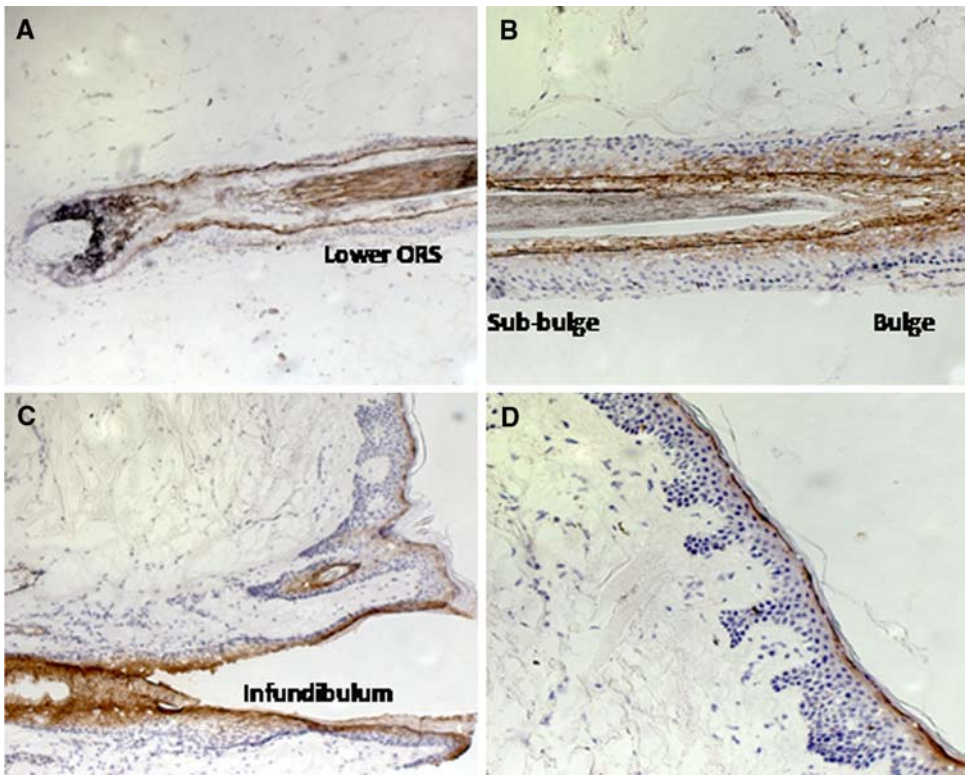

Fig. 9 CD24 immunoreactivity in a longitudinally sectioned human scalp HF (**a**–**c**) or vertically sectioned human scalp epidermis (**d**). ×400 (**a**), ×200 (**b**, **d**), or ×100 (**c**)

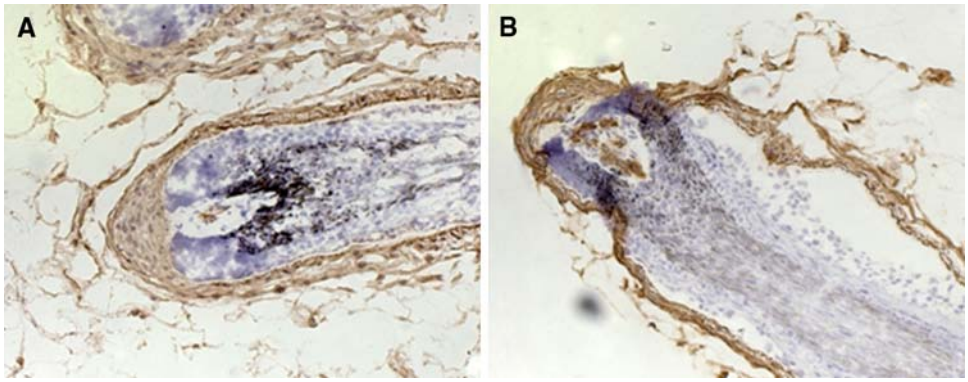

Fig. 10 CD90 immunoreactivity in a longitudinally sectioned HF (**a**, **b**) (×400)

Table 1 Summary of immunohistochemical staining patterns of human scalp epidermis and hair follicles

Location	Layer	Sublayer	CK 15	CD 24	CD 29	CD 34	CD 44	CD 49f	CD 56	CD 71	CD 90	CD 117	CD133	CD 200
Epidermis	B	Inter-ridge	+	–	+	+	–	+	–	+	–	–	–	–
		Rete Ridge	+	–	+	+	–	+	–	–	–	+	–	–
	SB		–	+	–	–	+	–	–	+	–	–	+	–
Infundibulum	ORS	B	+	–	+	–	+	–	–	–	–	+	+	++
		SB	–	+	–	–	+	–	–	–	–	–	+	–
	IRS		–	+	–	–	+	–	–	–	–	–	+	–
Bulge	ORS	B	++	–	+	–	+	±	–	–	–	–	+	++
		SB	–	+	–	–	+	–	–	–	–	–	+	–
	IRS		–	+	–	–	+	–	–	–	–	–	+	–
Sub-bulge	ORS	B	±	–	+	+	+	++	–	+	–	–	+	±
		SB	–	+	–	–	+	+	–	–	–	–	+	–
	IRS		–	+	–	–	+	–	–	–	–	–	+	–
Lower ORS	B		–	–	+	+	+	++	–	++	–	–	+	–
	SB		–	+	–	–	+	–	–	–	–	–	+	–
Bulb			–	+	+	–	+	+	+	–	–	–	+	–
Hair matrix			–	+	+	–	–	+	–	++	–	+	+	–
Dermal papilla			–	–	+	–	+	–	–	–	+	–	+	–

Staining intensities are: – no staining, ± marginally positive staining, + positive staining, and ++ strong positive staining

B basal cells, *SB* suprabasal cells, *ORS* outer root sheath, *IRS* inner root sheath, *DP* dermal papilla

Basal IFE and bulge cells are negative for CD24 and CD71

CD71 (transferrin receptor) is a 95 kDa type II homodimeric transmembrane glycoprotein expressed on proliferating cells (Hentze and Kuhn 1996; Trowbridge et al. 1993). CD71 staining was found in the outermost layer of the lower and sub-bulge ORS, as well as the hair matrix (Fig. 8e). Positive staining was also found in the basal and suprabasilar IFE, but not in the rete ridges (Fig. 8d).

CD24 (heat stable antigen or nectadrin) is a small (35–45 kDa) and highly glycosylated protein that has been implicated in cell adhesion and signaling (Kadmon et al. 1992; Kay et al. 1990). CD24 staining was found in the bulge ORS and IRS, the entire length of the companion layer, and hair matrix but not in the DP (Fig. 9). CD24-positive cells were also found in the stratum spinosum and rarely in stratum granulosum (Fig. 9d). CD24 staining did not colocalize with CK15 staining in the bulge (Fig. 2c) or sub-bulge (Fig. 2d).

CD90 is a specific DP stem cell surface marker

CD90 is a 25–35 kDa glycosylphosphatidylinositol-binding glycoprotein expressed on human bone marrow and cord blood $CD34^{+}$ hematopoietic progenitor cells, as well as mesenchymal stem cells (Craig et al. 1993; Morrison and Weissman 1994). CD90 staining was positive in the DP (Fig. 10), while other regions of the HF were negative (data not shown).

Other markers

We also examined the expression pattern of CD44, CD56, and CD133, all previously implicated markers of hair follicle stem cells (Florek et al. 2005; Shmelkov et al. 2005; Yin et al. 1997). We observed that CD44 and CD133 were broadly expressed in the stratum granulosum, IRS, ORS, and DP. No staining was detected in the basal IFE. CD56 staining was restricted to the bulb. The results for these markers as well as for those noted above are summarized in Table 1.

Discussion

To date, it remains unclear whether the IFE, HF, and SG are derived from unique stem cell progenitors or a common multipotent stem cell (Watt and Hogan 2000). Evidence in support of a common source includes the fact that bulge stem cells can differentiate into the epidermis, ORS, IRS, hair shaft, and SG (Ma et al. 2004). Furthermore, a previous study has shown that bulge stem cells can migrate to the epidermis (Morris et al. 2004). Conversely, when IFE stem cells were grafted into an empty HF, they differentiated to form a normal hair (Reynolds and Jahoda 1992). Although a number of markers have been utilized to identify epidermal and hair follicle stem cells, none of them have proven specific enough to allow researchers to distinguish between the unique stem cell populations found in the IFE and bulge.

In the present study, we freshly isolated human scalp skin and utilized a unique whole mounting method to more accurately assess the expression pattern of various cell surface markers previously implicated in epidermal or hair regeneration. Our study revealed that CK15, CD29, and CD49f are present both in the basal IFE and the bulge ORS. CK15 has been postulated to be a marker for bulge stem cells (Liu et al. 2003; Lyle et al. 1998). We found that CK15 stained the ORS of the upper- and middle-third of the follicle at the level of the arrector pili muscle, a zone that includes the putative bulge region (Figs. 1, 2). In addition, we found CK15 expressed in the basal IFE. This is in partial agreement with the results of Lyle et al., who found CK15 preferentially localized to the HF bulge although the upper portion of the follicle and overlying epidermis were negative in their study. Very recently, another report suggested that CK15 is occasionally expressed in the basal IFE (Inoue et al. 2009). Given its expression in the mitotically active basal layers of the IFE and HF, CK15 expression may be a proxy for the uncommitted state. Unfortunately, the utility of CK15 remains questionable since sorting requires cell surface markers and CK15 localizes to the cytoplasm.

CD29 (β1 integrin) and CD49f (a6 integrin) are putative markers of IFE stem cells (Brakebusch et al. 2000; Li et al. 1998). Integrins not only mediate adhesion to the underlying extracellular matrix, but they also regulate the initiation of terminal differentiation (Adams and Watt 1989; Watt et al. 1993). This may occur through a loss of function whereby down-regulation of integrin expression ensures that committed keratinocytes are selectively expelled from the basal layer (Adams and Watt 1990; Hotchin et al. 1995). In our study, we found CD29 expression in the basal IFE, and throughout the follicular ORS (Figs. 3, 4). Similarly, CD49f was expressed in the basal IFE, although its expression in the ORS was restricted to the bulge to bulb region (Figs. 3, 4). Although our results are consistent with previous findings, it appears that CD29 and CD49f cannot be used as specific markers to distinguish between bulge and basal IFE stem cells. Notwithstanding, these two markers may find utility when attempting to isolate basal IFE from primary skin preparations with dissociated epidermis. Caution should be exercised in employing CD29 and CD49f as sorting targets, however, as any remaining intrafollicular epithelia may result in inclusion of bulge stem cells.

More recently, studies using RNA laser capture microdissection and microarray analysis demonstrated that label-retaining human bulge stem cells are CD200 positive (Ohyama et al. 2006). The exact role of CD200 in bulge stem cell biology remains unclear. Since the CD200 receptor is involved in conferring immune privilege, CD200 may protect against immunological attack during inflammation, thereby helping to preserve this precious reservoir of regenerative stem cells (Rosenblum et al. 2004, 2006; Yu et al. 2005). Our results confirmed that bulge ORS cells express CD200 but we also found that this marker is not expressed in the basal IFE (Fig. 4). We also observed CD200 staining in the suprabulge ORS (infundibulum and SG duct) when examining longitudinal sections. Based on our results, CD200 is able to distinguish between basal IFE and bulge stem cells, although CD200 does not appear to be bulge-specific. Unfortunately, in our initial attempts as well as those of Ohyama et al. (personal communication) to utilize CD200 for FACS sorting of bulge stem cells were unsuccessful. We are currently investigating other techniques, such as serial magnetic sorting to enhance its detection.

CD34 has been shown to be expressed in mouse hair follicle stem cells, but its expression pattern in humans remains controversial. Ohyama et al. (2006) showed that CD34 was expressed in the non-bulge ORS, while Raposio et al. (2007) reported CD34 expression was in the bulge. Our data showed a notable absence of CD34 positive staining in the bulge region, although we did detect CD34 expression in the ORS in a zone marked superiorly by the arrector pili attachment and inferiorly by the matrix cells

(Fig. 7). Our immunostaining studies confirmed that CD34 positive cells were located below the bulge zone (Figs. 2, 4). Furthermore, the CD34 positive sub-bulge population was found to be CD200 and CK15 negative, indicating that they may represent transient amplifying cells or progeny of bulge stem cells. This is consistent with the positive CD34 staining observed in the basal IFE (Fig. 4), a finding not previously reported to date. The expression of CD34 on basal IFE stem cells could enhance their proliferation and block their differentiation and adhesion (Hoang et al. 2009; Poblet et al. 2006). Altogether, our data suggest that the combination of CD34 and CD200 may together allow for discrimination between basal IFE and bulge stem cells and can be used as a negative selection marker for bulge stem cell isolation.

CD117, a type III tyrosine kinase receptor, and its ligand stem cell factor play an important role in pigmentation (Giebel and Spritz 1991). Our data revealed that CD117 was expressed in the outermost layer of the infundibulum and hair matrix (Fig. 8), both known reservoirs of melanocyte stem cells (Grichnik et al. 1996). To our surprise, we found CD117 expression could be detected in the basal IFE in a pattern restricted to the rete ridges (Fig. 8c), the reservoir for IFE stem cells (Ghazizadeh and Taichman 2005; Lavker and Sun 1982, 2000). Previous studies have shown that CD117-reactive cells do not co-localize with keratinocyte markers (Grichnik et al. 1996). Further double-labeling studies are underway to clarify the identity of the CD117 positive rete ridge signal.

We investigated a number of other markers previously implicated in hair follicle and epidermal regeneration (Table 1). For example, we pursued CD71 since it is only expressed in cycling cells (Li and Kaur 2005) and CD24 which was previously shown to be upregulated during keratinocyte differentiation (Magnaldo and Barrandon 1996). Indeed, we found that both CD71 and CD24 could be used as negative selection markers for ORS bulge stem cells. To isolate bulge stem cells from whole hair follicles, some investigators have elected to microdissect the bulge region followed by plating in tissue culture. While useful, this method remains subjective since the bulge lacks distinct morphology in humans. Moreover, the bulge region is located close to the bulb during telogen/catagen, suggesting that the dermal papilla stem cells may be a source of contamination especially since these cells cycle faster than bulge stem cells. Therefore, we examined the HF for expression of CD90 since this marker has previously been shown to be expressed in mesenchymal stem cells (Bosch et al. 2006) and fibroblasts (Nazareth et al. 2007).

We found that CD90 staining was restricted to the DP stem cells in the bulb region, suggesting that this marker could be used to negatively select bulge stem cells from fresh follicular preparations.

To date, it has been challenging to derive pure populations of human stem cells involved in hair follicle regeneration. In the absence of this, clinical exploitation of HF stem cells for the treatment of alopecia is unlikely to become commercially viable. The first step in obtaining pure populations involves development of sorting strategies based on cell surface markers unique to each cell population. In this study, we developed a novel method to whole mount single HFs that allowed us to carry out more thorough in situ characterization studies. Our study revealed several cell surface markers localized uniquely to different stem cell populations involved in hair regeneration. The markers tested in this study may allow pursuit of cell sorting and purification strategies in order to determine unique molecular signature of each stem cell population.

Acknowledgments We thank Dr. Jian Zhang for help in designing several of the primers used in this study. This work was supported by a generous gift from the Minnesota Jewish Foundation (2006) and an ASDS Cutting Edge Grant (2006 and 2007), both of which were awarded to Basil M. Hantash.

Conflict of interest statement The authors state no conflict of interest.

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