The cell-surface marker MTS24 identifies a novel population of follicular keratinocytes with characteristics of progenitor cells

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We describe a novel murine progenitor cell population localised to a previously uncharacterised region between sebaceous glands and the hair follicle bulge, defined by its reactivity to the thymic epithelial progenitor cell marker MTS24. MTS24 labels a membrane-bound antigen present during the early stages of hair follicle development and in adult mice. MTS24 co-localises with expression of α6-integrin and keratin 14, indicating that these cells include basal keratinocytes. This novel population does not express the bulge-specific stem cell markers CD34 or keratin 15, and is infrequently BrdU label retaining. MTS24-positive and -negative keratinocyte populations were isolated by flow cytometry and assessed for colony-forming efficiency. MTS24-positive keratinocytes exhibited a two-fold increase in colony formation and colony size compared to MTS24-negative basal keratinocytes. In addition, both the MTS24-positive and CD34-negative subpopulations were capable of producing secondary colonies after serial passage of individual cell clones. Finally, gene expression profiles of MTS24 and CD34 subpopulations were compared. These results showed that the overall gene expression profile of MTS24-positive cells resembles the pattern previously reported in bulge stem cells. Taken together, these data suggest that the cell-surface marker MTS24 identifies a new reservoir of hair follicle keratinocytes with a proliferative capacity and gene expression profile suggestive of progenitor or stem cells.

KEY WORDS: Hair follicle, Progenitor cell, Stem cell, Epidermis, Keratinocyte, Mouse

INTRODUCTION

The epidermis is a complex tissue consisting of a stratified squamous epithelium (interfollicular epidermis), hair follicles and glandular structures that function together as an organism’s main barrier against the external environment. Because of continual physical, chemical and biological damage from the environment, the epidermis undergoes regular self-renewal. Epidermal stem cells, which form the basis of this system, reside in several locations including the interfollicular epidermis, sebaceous glands, and hair follicles (Niemann and Watt, 2002; Fuchs et al., 2004; Moore and Lemischka, 2006). Hair follicles are multilayered epidermal appendages of several concentric layers that undergo a carefully regulated growth cycle divided into phases of active growth (anagen), regression (catagen) and rest (telogen) (Hardy, 1992).

Hair follicle stem cells persist throughout the lifetime of the organism and are located in a well-protected and nourished niche called the bulge (Cotsarelis et al., 1990; Morris and Potten, 1999). The bulge is localised to the lowest permanent part of the outer root sheath (ORS) epithelium defined by the insertion site of the arrector pili muscle (Cotsarelis et al., 1990; Morris and Potten, 1999). Bulge epidermal cells are characterised by their relatively undifferentiated ultrastructure and infrequently dividing (quiescent) nature (Cotsarelis et al., 1990; Lyle et al., 1998; Morris and Potten, 1999; Akiyama et al., 2000; Tumbar et al., 2004). A small number of bulge-associated stem cells proliferate at the onset of hair growth and during wound repair (Lyle et al., 1998; Tumbar et al., 2004). Bulge cells in adult mice are multipotent: they give rise to all epithelial cell lineages within the intact follicle during normal hair cycling, and can be recruited to transiently contribute to the epidermis in response to stimuli such as wounding (Taylor et al., 2000; Oshima et al., 2001; Morris et al., 2004; Blanpain et al., 2004). It is known that epidermal stem cells are capable of asymmetric cell division to produce both quiescent daughter stem cells and more-frequently dividing progenitor cells, which are committed along a differentiation pathway. Despite this multipotent phenotype, recent studies demonstrate that hair follicle-associated keratin 15+ stem cells are not required for normal epidermal homeostasis (Ito et al., 2005).

Currently, there are several methods to experimentally distinguish epidermal stem cells from the cycling transit amplifying cells. One approach is to pulse-label neonatal mice repeatedly with injections of [3H]thymidine or 5-bromo-2’-deoxyuridine (BrdU). Using this method, all the actively dividing cells in the epidermis are labelled at a time when the skin is hyperproliferative. This pulse is followed by a long chase period (4-10 weeks) during which the [3H]thymidine- or BrdU-label is lost through proliferation-associated dilution. In contrast, infrequently dividing stem cells retain the label and are therefore called label-retaining cells (LRC) (Bickenbach, 1981; Cotsarelis et al., 1990; Bickenbach and Chism, 1998; Lavker and Sun, 2000).

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A second approach to distinguish epidermal stem cells from transit amplifying cells in humans involves analysis of the proliferative potential of single cultured cells. Analysis of the resulting epidermal clones led to classification of keratinocytes into stem-like, highly proliferative holoclones and more abortive meroc- and paraclone colonies (Barrandand and Green, 1987). Several studies have shown that LRC isolated from skin of adult mice (Morris and Potten, 1994) or rats (Pavlovitch et al., 1991; Kobayashi et al., 1993) are also clonogenic in culture. These follicular keratinocytes were highly proliferative, particularly in the rat where the hair follicle bulge region contains predominantly (95%) clonogenic keratinocytes (Kobayashi et al., 1993; Oshima et al., 2001). The multipotentiality of individual mouse pelage or rat vibrissal bulge stem cells was demonstrated by mouse skin transplantation, where clonally derived cells were able to give rise to new hair follicles (Blanpain et al., 2004) or contribute to endogenous developing follicles (Claudinot et al., 2005).

Epidermal stem cells have also been distinguished from transit amplifying cells by their unique cell phenotype. Initially, human epidermal stem cells and transit amplifying cells were distinguished by differential expression of integrins and keratins. Human epidermal stem cells revealed a higher expression of \( \beta 1 \), \( \alpha 2 \), \( \alpha 3 \) and \( \alpha 6 \)-integrin compared to transit amplifying cells (Jones et al., 1995; Tani et al., 2000; Akiyama et al., 2000; Braun et al., 2003). Murine epidermal stem cells have been characterised by a strong expression of keratin 15 (K15) (Liu et al., 2003; Morris et al., 2004), although this marker may not be exclusive to stem cells in all situations (Amoh et al., 2005). Expression of \( \alpha 6 \)-integrin (in combination with a low expression of the transferrin receptor CD71) and K19 have been correlated with \(^{3}H\)thymidine-label-retaining-cells, indicating that these markers can identify murine epidermal stem cells (Michel et al., 1996; Tani et al., 2000). Another approach has been to examine candidate cell-surface markers that identify stem cells in other tissues. The cell-surface glycoprotein CD34 is expressed on early hematopoietic progenitor cells, and its use in the purification of stem cells for bone marrow transplants has been well established (Brown et al., 1991; Krause et al., 1994). More recently, CD34 was shown to be expressed in the hair follicle bulge of murine skin and CD34-positive cells, purified by fluorescence-activated cell sorting (FACS), were shown to have clonogenic potential in vitro (Trempus et al., 2003).

Recent evidence suggests that epidermal keratinocytes are capable of recruiting hematopoietic precursors and supporting development of a thymic microenvironment (Clark et al., 2005). These data suggest potential functional and phenotypic links may exist between progenitor cells in epidermal and thymic epithelia. Several years ago, a specific monoclonal antibody marker was described for epithelial progenitor cells in the mouse thymus. This marker, MTS24, identified a glycoprotein with a peptide backbone of \(~80\) kD, which was expressed on a rare subset of epithelial cells in the adult thymus (Gill et al., 2002; Bennett et al., 2002). During the early embryonic development of the thymus, a large proportion of thymic epithelial cells are reactive for MTS24. Transplantation of purified fetal MTS24-positive thymic epithelial cells under the kidney capsule generated a normal microenvironment, indicating that MTS24-positive thymic epithelial cells comprise a population of precursor cells capable of recruiting hematopoietic precursors and giving rise to a fully functional thymic epithelium.

Here, we report that the cell-surface marker MTS24 identifies a previously uncharacterised population of hair follicle keratinocytes located between the bulge and the sebaceous glands. MTS24 reactivity is first detected in the early stages of hair follicle development, and is increased during hair growth. MTS24-positive keratinocytes are distinct from the epidermal stem cells located in the bulge, but exhibit increased colony-forming efficiency in culture versus normal basal keratinocytes. Furthermore, the gene expression profile of MTS24-positive keratinocytes resembles the pattern previously reported for epidermal bulge stem cells. Our results suggest that the MTS24-positive keratinocytes represent an important new committed progenitor or stem cell compartment within the hair follicle.

**MATERIALS AND METHODS**

**Experimental mice**

To assess and compare MTS24 in different mouse strains we obtained mice with normal hair development (C57Bl/6 mice and Balb/c) and mice with abnormal hair development (nude and SKH-1 hairless mice) at 6-8 weeks of age from Charles River (Maastricht, The Netherlands). The SKH-1 mice are an uncharacterised hairless strain of mice that go through one hair cycle, after which they lose their fur and become hairless.

Mice were housed in the animal facility of the Erasmus University Medical Centre under a 12-hour light–dark cycle at 23°C/60% humidity and given food and water ad libitum in accordance with the university’s ethical committee guidelines on animal care. At Cancer Research UK all mouse husbandry and experimental procedures were conducted in compliance with the CR-UK animals ethics committee. The K14\(\Delta\beta\)-cateninER transgenic mice were generated as described previously (Lo Celso et al., 2004). The K14\(\Delta\beta\)-cateninER transgene was activated by topical application of 4-hydroxytamoxifen (4OHT; Sigma) to a clipped area of dorsal skin (1 mg per mouse; 3 treatments/week).

**BrdU labelling**

To generate label-retaining cells (LRC), we used the protocol as described by Bickenbach and colleagues (Bickenbach et al., 1986; Bickenbach and Chism, 1998). Ten-day-old mice were injected with 50 mg/kg body weight 5-bromo-2'-deoxyuridine (BrdU; 20 μl of 12.5 mg/ml BrdU) every 12 hours for a total of four injections, to label mitotic cells.

**Tissue preparation**

Mice were killed with CO\(_2\). Dorsal skin and tail skin were embedded in tissue-tek O.C.T. compound (Sakura Finetek Europe). Frozen sections (5-7 μm) were fixed for 30 minutes in formaldehyde (1% in PBS). Wholomegns from tail skin were prepared as described by Braun and colleagues (Braun et al., 2003). Epidermal wholemounts were fixed for a minimum of 10 minutes to 2 hours in formaldehyde (1% in PBS).

**Antibodies**

Antibodies against the following antigens were used: MTS24 (Gill et al., 2002), keratin 17 (kind gift from P. Coulombe) (McGowan and Coulombe, 1998), keratin 10 (Covance), keratin 14 (Covance), keratin 15 (kind gift of I. Leigh) (Waseem et al., 1999), FITC-conjugated anti-BrdU (Dako), mouse anti-BrdU (BD Biosciences), sheep anti-BrdU (Biodesign), FITC-conjugated rat anti-human \( \alpha 6 \)-integrin and R-phycoerythrin (RPE)-conjugated rat anti-human \( \alpha 6 \)-integrin (CD49f, BD Biosciences), CD34 and biotinylated CD34 (BD Biosciences) and RPE-conjugated rat IgG2a isotype control (BD Biosciences). Secondary antibodies included donkey anti-rat Cy3 (Jackson ImmunoResearch Laboratories), goat anti-rabbit FITC (Zymed), rabbit anti-rat biotin (Dako), RPE-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories), streptavidin-conjugated RPE (Caltag) and allophycocyanin (APC)-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories). Additional species-specific secondary antibodies were conjugated to AlexaFluor 488, AlexaFluor 594 (Molecular Probes) or Cy3 (Amersham Pharmaica Biotech) prior to use. Sections were counterstained with either 4',6-diamidino-2-phenylindole (DAPI) or ToPro3 (Molecular Probes) to visualise nuclei.

**Fluorescence staining of tissue sections and epidermal sheets**

Following fixation, frozen sections were preincubated for 30 minutes with 5% normal human serum (NHS, obtained from LUMC blood bank) to block non-specific antibody binding. Tissues were incubated with rat anti-mouse
MTS24 (as hybridoma cell culture supernatant diluted 1:50 in PBS/1% BSA/1% NHS) for 1 hour at room temperature (RT), followed by incubation with donkey anti-rat Cy3 (1:1000 in PBS/1% BSA with 1% normal mouse serum added) at RT for 1 h. Tissues were either mounted with Vectashield (Vector Laboratories) containing DAPI (5 μg/ml) and coverslipped prior to fluorescence microscopy (Leica DM RXA) or co-stained with antibodies against keratin 17 (K17), keratin 15 (K15), keratin 14 (K14), keratin 10, α6-integrin, Brdu or CD34. To detect Brdu-labelled cells, MTS24-stained tissues were incubated with 2 M HCl for 30 minutes. This reaction was stopped by adding 1 M TRIS solution for 5 minutes. Tissues were incubated with FITC-conjugated anti-BrdU (1:25) overnight at 4°C. To avoid cross-reactivity when performing dual immunofluorescence for MTS24 and CD34, MTS24 was visualized with AlexaFluor 488-conjugated goat anti-rat, and CD34 was directly conjugated to Cy3. For co-immunolabelling of CD34 and K17, tissues were fixed in acetone for 10 minutes and preincubated for 30 minutes with 5% NHS. Tissues were incubated with rat anti-mouse CD34 (1:50 in PBS/1% BSA/1% NHS) and then incubated with donkey anti-rat Cy3 (1:1000 in PBS/1% BSA with 1% normal mouse serum added) following co-staining with K17 as described above. Epidermal wholemounts were preincubated, labelled, mounted and visualised as previously described (Braun et al., 2003).

**FACS and clonogenicity assay**

Keratinocytes were isolated and cultured from dorsal skin of adult C57Bl/6 mice essentially as reported previously by Romero et al. (Romero et al., 1999), incorporating the modifications described by Silva-Vargas et al. (Silva-Vargas et al., 2005). To dual-label keratinocytes for α6-integrin/MTS24 or α6-integrin/CD34, cell suspensions were incubated with either MTS24 (diluted 1:50 or 1:100) or biotinylated CD34 antibody for 20 minutes at 4°C and then washed with PBS. Cells were subsequently incubated with RPE-conjugated donkey anti-rat IgG (to detect MTS24) or streptavadin-conjugated RPE (to detect CD34). Cells were then washed and blocked for 10 minutes in normal mouse serum (1:100) followed by incubation with FITC-conjugated rat anti-human α6-integrin (Trempeus et al., 2003). The α6 antibody was used to select for basal keratinocytes, thus eliminating suprabasal (differentiating) keratinocytes and non-keratinocytes from the population collected. Cell viability was assessed by trypan blue staining. Dead cells and cells with high forward and side scatter were gated out. Cells were sorted into supplemented, calcium-free FAD media containing 10% foetal bovine serum (Invitrogen) using a FACSVantage machine (Becton Dickinson). Sorted populations were gated as follows: α6-integrin single+, α6+/MTS24+, or α6+/CD34+ dual positive. Sorting gates were drawn based upon staining intensity of single colour controls, and were excluded in all regions from overlapping with negative and/or isotype controls. One thousand keratinocytes were plated per 35 mm dishes, and grown for an additional 10-14 days, after which time the average number and surface area of colonies was assessed. Two-tailed unpaired t tests were performed with significance recognised with P<0.05 (GraphPad Software, San Diego).

To perform FACS analysis for MTS24 and CD34, cells were labelled with the primary antibody for MTS24 for 20 minutes at 4°C, washed twice in PBS and incubated in AlexaFluor 488 goat anti-rat IgG. Cells were then washed and blocked for 10 minutes in normal mouse serum (1:100) prior to incubation with a biotinylated CD34 antibody followed by further washing and incubation with streptavidin conjugated RPE.

**RNA isolation and quantitative real-time PCR**

We performed quantitative real-time PCR (Q-PCR) to determine the expression of a selection of genes that were expected to be up- or downregulated in hair follicle stem cells compared to non-stem cells (Tumbar et al., 2004; Morris et al., 2004; Claudinot et al., 2005). Using FACS (method described in previous section), we isolated α6-integrin single+, α6+/MTS24+ and α6+/CD34+ keratinocytes obtained from skin of C57Bl/6 mice (>100,000 cells per population). Total RNA was isolated from the sorted cells (average yield was 110 ng total RNA/100,000 cells) with the RNeasy Mini Kit (Qiagen) and mRNA amplification was performed with the MessageAmp II aRNA Amplification Kit (Ambion) using T7-o1igo-(dT) primers according to the manufacturer’s protocol. cDNA was synthesized from amplified RNA with iScript Select CDNA synthesis kit (Bio-Rad) using random priming. Q-PCR assays were performed on a MyIQ single colour real-time PCR (Bio-Rad) using SYBR Green Supermix (Bio-Rad). PCR reaction was carried out according to the following protocol: initial denaturation at 95°C (3 minutes) followed by 40 cycles of 95°C (15 seconds) and 58°C (20 seconds). Primer sequences can be provided on request. A melting curve was generated for each product to ensure the specificity of the PCR product. Threshold cycles (Ct values) were calculated using the MyIQ software (Bio-Rad). The reference gene beta-actin was used to normalise the Ct values of the genes of interest (ΔCt). Relative alterations (fold change) in mRNA expression levels in α6+/MTS24+ and α6+/CD34+ keratinocytes were calculated according to the algorithms 2−ΔΔCt=2−ΔCtα6+MTS24−/α6−CD34− and 2−ΔΔCt=2−ΔCtα6+MTS24−/2−ΔCtα6+CD34−, respectively. FACS isolation was performed in duplicate, and each Q-PCR reaction was performed in triplicate.

**RESULTS**

**MTS24 reactivity in murine hair follicles and interfollicular epidermis**

Immunofluorescent staining of tissue sections (Fig. 1A,B; red) or whole mounts (Fig. 1F,G; green) of tail skin from adult C57Bl/6 mouse showed bright staining of MTS24 in the hair follicle. MTS24 was predominantly found in a previously uncharacterised region of the hair follicle between the bulge and the sebaceous glands. The intensity of this staining decreased towards the lower part and upper part of the hair follicle, although labelling was occasionally seen in the infundibulum (the upper part of the hair follicle), as well as in the cells at the perimeter of the sebaceous gland (data not shown). A similar pattern of MTS24 labelling was found in dorsal skin from Balb/c (Fig. 1C-E), SKH-1 hairless mice (Fig. 1H-J) and nude mice (data not shown). Higher magnification revealed that MTS24 was primarily located on the membrane of hair follicle cells (Fig. 1E). However, along the inner hair shaft MTS24 staining was not membrane-bound but had a more smear-like appearance (Fig. 1H, asterisk). The hair follicle bulb did not show any MTS24 labelling (Fig. 1F). Negative controls did not show any MTS24 labelling (Fig. 1F).

The staining pattern of MTS24 in murine skin of 2-day-old SKH-1 mice (Fig. 1J) was further characterised using immunoelectronmicroscopy (Fig. 1K-N). A cross-sectional overview of the murine hair follicle (Fig. 1M) showed that MTS24 reactivity was found in both the outer root sheath (ORS; see also Fig. 1K) and the...
inner root sheath (IRS; see also Fig. 1L,N). Within the ORS, metabolising cells were found, characterised by the presence of heterochromatin (Fig. 1K,M; asterisks). These cells showed membrane-bound staining for MTS24, as indicated by the gold particles associated with their cell membrane (Fig. 1K, arrowheads). Within the IRS, apoptotic cells were found (Fig. 1L,M; crosshatch). These apoptotic cells also showed membrane-bound MTS24 labelling (Fig. 1L). More centrally within the IRS, many tightly packed membranes of dead cells were found, which the MTS24 antibody also labelled (Fig. 1N). This observation correlates with the smearing pattern of MTS24 as shown in Fig. 1H. Taken together, these data indicate that MTS24 labels a membrane-bound antigen that is localised in a previously uncharacterised region of the murine hair follicle adjacent to the bulge.

**MTS24 and CD34 localisation during hair follicle development**

To investigate the relationship between MTS24 expression reactivity and murine hair follicle development, we analysed MTS24 labelling in murine dorsal skin at different stages of hair follicle development. The timepoints ranged from embryonal day 17 (E17), at which the largest group of hair follicles (non-tylotrich follicles) start to develop (Schmidt-Ullrich and Paus, 2005), to postnatal day 8, by which time all hair follicles are fully developed. Recently, expression of the cell-surface glycoprotein CD34 has been reported in keratinocytes localised in the bulge region of the adult hair follicle (Tremps et al., 2003). To permit comparison of CD34 and MTS24 reactivity during both normal and aberrant hair follicle development, we labelled frozen sections of dorsal skin from Balb/c or SKH-1 mice with antibodies to MTS24 or CD34, and co-stained with keratin 17 (K17), a marker exclusively expressed in hair follicles of normal skin (Panteleyev et al., 1997). We first observed weak co-localisation of MTS24 and K17 in the developing hair follicle at E17 (data not shown). At E20.5 MTS24 staining was intense and showed co-localisation with K17 expression in the developing hair follicle (Fig. 2A, upper panel). As hair follicle development progressed further, both MTS24 and K17 labelling increased in intensity. At day 2 after birth, MTS24 was found in the entire upper hair follicle as well as in the interfollicular epidermis, both in the SKH-1 mouse (Fig. 2A, second panel, arrowhead). In all cases, the interfollicular labelling was specifically localised to areas where new hair follicles were developing. From 7-8 days after birth, MTS24 labelling became completely restricted to the hair follicle (Fig. 2A, third panel).
In contrast to MTS24, which was first detectable at E17 and was clearly visible at E20.5 during hair follicle development (Fig. 2A), hair follicles in neonatal Balb/c mice up to 4 days of age (Fig. 2B) failed to show labelling for CD34. However, at 6 days after birth CD34 labelling was observed in the bulge region of the hair follicle (Fig. 2C). In contrast to MTS24, in adult SKH-1 hairless mice no labelling for CD34 was ever observed in the hair follicle bulge (data not shown). These data indicate that MTS24 is present at an earlier stage than CD34 expression in hair follicle development.

It has been reported that activation of β-catenin in the epidermis of K14ΔNβ-cateninER adult transgenic mice by topical application of 4-hydroxytamoxifen (4OHT) results in the formation of ectopic hair follicles from sebaceous glands, interfollicular epidermis and pre-existing hair follicles (Van Mater et al., 2003; Lo Celso et al., 2004; Silva-Vargas et al., 2005). This expansion in the number of hair follicles is associated with a dramatic increase in the percentage of CD34-reactive, follicular stem-like cells in the skin (Silva-Vargas et al., 2005). To assess whether MTS24 reactivity is similarly increased during de novo hair follicle formation, we stained frozen sections of dorsal tissue collected from K14ΔNβ-cateninER transgenic mice following thrice-weekly treatment with 4OHT for 21 days. MTS24 reactivity was found in ectopic follicles formed from both interfollicular epidermis and pre-existing follicles (Fig. 2D). During the early stages of follicle neogenesis, MTS24 was present throughout the developing follicle (data not shown), reminiscent of the staining pattern observed during embryonic development (Fig. 2A). As the follicles developed further, MTS24 began to be restricted to a mid-region of the K17-positive follicle (Fig. 2E). In addition, we assessed the localisation of MTS24 labelling in normally cycling hairs (see Fig. S1 in the supplementary material), demonstrating that expression is increased during anagen. In summary, these findings indicate that MTS24 reactivity is increased during de novo hair follicle formation and during the growth (anagen) phase of the hair cycle.

MTS24 localisation with described stem cell markers

We used several described markers of the epidermal stem cell compartment to examine their co-localisation with MTS24. K14 is expressed in all keratinocytes in the basal layer of interfollicular epidermis as well as in the outer root sheath of the hair follicle (Fig. 3A). Immunolabelling of frozen sections of dorsal epidermis showed that K14 and MTS24 co-localise within the hair follicle, demonstrating that MTS24-positive cells are keratinocytes (Fig. 3A-C). Label-retaining cells tend to be clustered in the hair follicle bulge (Cotsarelis et al., 1990), a region that also expresses high levels of the markers CD34, keratin 15, and α6-integrin (Lyle et al., 1998; Tremps et al., 2003; Morris et al., 2004). In wild-type dorsal mouse skin, expression of CD34 (Fig. 3D, red, arrowhead) in the hair follicle was adjacent to, but did not co-localise with MTS24 (Fig. 3D, green). We stained frozen sections of dorsal tissue collected from K14ΔNβ-cateninER transgenic mice following thrice-weekly treatment with 4OHT for 21 days. MTS24 reactivity was found in ectopic follicles formed from both interfollicular epidermis and pre-existing follicles (Fig. 2D). During the early stages of follicle neogenesis, MTS24 was present throughout the developing follicle (data not shown), reminiscent of the staining pattern observed during embryonic development (Fig. 2A). As the follicles developed further, MTS24 began to be restricted to a mid-region of the K17-positive follicle (Fig. 2E). In addition, we assessed the localisation of MTS24 labelling in normally cycling hairs (see Fig. S1 in the supplementary material), demonstrating that expression is increased during anagen. In summary, these findings indicate that MTS24 reactivity is increased during de novo hair follicle formation and during the growth (anagen) phase of the hair cycle.

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DEVELOPMENT

CD34 expression was found directly beneath the MTS24 region (Fig. 3F, red versus green). In whole mounts of tail epidermis, labelling for K15 (Fig. 3G, green) and MTS24 (Fig. 3H, red) did not co-localise (Fig. 3I). A negative control for keratin 15 (incubation without the primary antibody) showed that the intense green staining within the sebaceous gland was background staining due to use of an anti-mouse secondary antibody (Fig. 3J). α6-Integrin expression was very bright throughout the entire hair follicle, including the bulge and the region where MTS24 was detected (Fig. 3K, arrowhead).

To investigate whether MTS24-positive keratinocytes are rarely dividing cells, we injected SKH-1 and CBAxC57Bl/6 10-day-old mice repeatedly with BrdU to generate label-retaining cells (LRC). In SKH-1 mice MTS24-positive cells were BrdU labelled at one day post-injection (Fig. 3L). After a chase period of 6 weeks, LRC were still occasionally found within the population of MTS24-positive cells, although the majority of the cells have depleted their label (Fig. 3M). In CBAxSC57Bl/6 mice, after a 10 week chase, the region of the hair follicle that was MTS24 reactive contained some BrdU-positive cells, but most of the LRC were clustered in the bulge region of the follicle, beneath the MTS24-positive region of the follicle (Fig. 3N,O).

Taken together, these findings indicate that MTS24 labelling co-localised with expression of the basal keratinocytes markers α6-integrin and keratin 14 but not with the bulge-specific markers keratin 15 and CD34. BrdU label-retaining cells occasionally were found within the MTS24-positive cell population.

**MTS24-positive keratinocytes form large colonies with high efficiency in culture**

FACS analysis was performed to compare immunolabelling of keratinocytes incubated with isotype-specific control (Fig. 4A) or MTS24 (Fig. 4B) antibodies. In this representative experiment (Fig. 4B), 4.1% of the keratinocytes were gated as MTS24-positive; typically in our experiments the MTS24-positive subpopulation of keratinocytes ranged from 4% to 8%. We used FACS enrichment to

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**Fig. 3. MTS24 co-staining with described markers for epidermal stem cells.** (A-C) Frozen section of dorsal skin showing expression of keratin 14-Cy3 (A; red) and MTS24-FITC (B; green) and merged (C) in hair follicle. Arrowhead highlights co-localisation of keratin 14 expression and MTS24 labelling. (D-F) Expression of CD34-Cy3 (D; red) and MTS24-FITC (E; green) and merged (F) in dorsal skin. Note that CD34 expression (arrowhead) was found in a different location within the hair follicle than MTS24-staining (asterisk). (G-I) Wholemount of tail epidermis showing no co-localisation between keratin 15-FITC (G; green) and MTS24-Cy3 (H; red) labelling within the hair follicle. Merged image is shown in (I). (J) Labelling with MTS24-Cy3 (red) and FITC anti-mouse (green) secondary antibody alone, shows that staining of sebaceous gland is non-specific in (G,I). (K) Wholemount of tail epidermis showing α6 integrin-FITC (green) and MTS24-Cy3 (red) co-staining (arrowhead). SKH-1 (L,M) or wild-type (N,O) neonatal mice received repeated injections with BrdU to generate label-retaining cells. Frozen sections (L-N) or tail wholemounts (O) were collected at 1 day (L), 6 weeks (M) or 10 weeks (N,O) after the last injection with BrdU. Tissue was labelled for BrdU-FITC (green) and MTS24-Cy3 (red). Nuclear counterstain was DAPI (L,M,N; blue). Arrowheads indicate BrdU label-retaining cells (L-O). In each panel the bulge area is bracketed. SG, sebaceous gland; HF, hair follicle; IFE, interfollicular epidermis. Scale bars: 50 μm.
determine whether MTS24-positive keratinocytes possess a high in vitro proliferative potential, a well-established characteristic of epithelial stem cells (Kobayashi et al., 1993). Data are shown from a representative experiment containing at least four replicates for each sort condition indicated; this experiment was repeated three times with similar results (Fig. 4C-F). Three groups of sorted keratinocytes were collected and seeded at clonal density: the unfractionated 'all sorted' population of undifferentiated cells with low forward and side scatter, α6+/MTS24+ cells (6.2% of undifferentiated cells) and α6+/MTS24– cells (48.1% of undifferentiated cells) (Fig. 4C). After 14 days in culture, all three populations formed colonies, however the α6+/MTS24+ keratinocytes gave rise to colonies with the greatest efficiency (Fig. 4D,E). In addition, there was enrichment for larger colonies from the α6+/MTS24+ population (Fig. 4D,E). Statistical analysis demonstrated significant differences in colony-forming efficiency (P<0.0003) and the average area of the colonies (P<0.0003) between the α6+/MTS24+ and α6+/MTS24– fractions (Fig. 4E, asterisks). We quantified the percentage of the total number of colonies based upon the size of the colonies (Fig. 4F). The results showed that α6+/MTS24+ keratinocytes form abortive colonies (colony area <1 mm²) significantly less frequently than α6+/MTS24– keratinocytes (Fig. 4F; P<0.0001). In contrast, the α6+/MTS24+ fraction produced significantly more large colonies than α6+/MTS24– keratinocytes (colony area 3-4 mm², P<0.0008; colony area >4 mm², P<0.02) (Fig. 4F). These results indicate that MTS24+ basal keratinocytes possess a higher degree of proliferative potential when compared to normal basal keratinocytes.

**CD34 and MTS24 identify distinct clonogenic subpopulations of basal keratinocytes**

A population of clonogenic CD34+positive basal keratinocytes has been reported in the hair follicle bulge (Trempus et al., 2003). To assess whether CD34+ keratinocytes represent a unique population from MTS24+ cells, keratinocytes isolated from C57Bl/6 mouse dorsal skin were labelled with antibodies to CD34 and MTS24. FACS analysis identified distinct CD34+/MTS24+ (0.67%) and CD34–/MTS24+ (4.4%) populations of cells (Fig. 5A). We were unable to detect any CD34+/MTS24+ dual-positive keratinocytes. To directly compare the colony-forming potential of CD34+ versus MTS24+ basal keratinocytes, cells isolated from dorsal skin of adult C57Bl/6 mice were immunolabelled with antibodies against MTS24/α6-integrin or CD34/α6-integrin. FACS sorting and culture conditions were performed as described in Fig. 4. Data shown are representative of three separate experiments with six replicates for each sort condition indicated (Fig. 5B-E). After 14 days in culture, sorted α6+/CD34+ keratinocytes gave rise to the largest colonies which were composed of mainly small, apparently undifferentiated keratinocytes. The α6+/MTS24+ colonies were intermediate in terms of colony size and relative fraction of small colonies.
keratinocytes, while the α6-single positive fractions predominantly generated small colonies composed of mostly large, differentiated keratinocytes (Fig. 5B). Both the α6+/CD34+ and α6+/MTS24+ subpopulations had increased relative colony-forming efficiency compared with the unfractonated ‘all sorted’ population (Fig. 5C), although the α6+/CD34+ sorted keratinocytes were more efficient at primary colony formation (Fig. 5C).

We next wished to determine whether isolated, sorted, and cultured individual colonies were capable of giving rise to secondary colony cultures with high efficiency. Individual colonies from α6+/MTS24+ and α6+/CD34+ sorted keratinocytes were cultured for 10 days, isolated by ring cloning, and re-plated on secondary dishes for an additional 14 days. Both progenitor cell subpopulations efficiently generated secondary colonies following serial passage (Fig. 5D), and average colony size was not significantly different between bulge-derived CD34+ stem cells and MTS24+ cells (Fig. 5E).

**Gene expression profiling of MTS24 versus CD34 basal keratinocytes**

Microarray studies have revealed that hair follicle stem cells exhibit a specific gene expression profile compared to non-bulge basal keratinocytes (Morris et al., 2004; Tumbar et al., 2004; Claudinot et al., 2005). Based on these data, we selected 13 genes that were described to be up- or downregulated in hair follicle stem cells compared to non-stem cells. Using Q-PCR we studied the expression profile of these selected genes in FACS-sorted α6+/MTS24+ and α6+/CD34+ keratinocytes compared to α6+/MTS24− and α6+/CD34− keratinocytes. Average data are shown from two independent FACS sorting experiments and Q-PCR was performed in triplicate per sorted population (Fig. 6). In general, we observed that α6+/MTS24+ (Fig. 6, filled bars) and α6+/CD34+ (Fig. 6, hatched bars) keratinocytes showed a similar gene expression profile for genes whose expression is expected to be downregulated (Fig. 6, red bars) and for genes whose expression is expected to be upregulated (Fig. 6, green bars). For example, Dab2 (which encodes a Wnt-inhibitor) (Hocevar et al., 2003) and Eps8 (which encodes an EGF-pathway member) (Miyamoto et al., 1996), whose increased expression is associated with bulge stem cells, indeed show elevated expression in both α6+/MTS24+ and α6+/CD34+ keratinocytes. Genes involved in hair growth (Wnt3a) (Millar et al., 1999) and hair follicle differentiation (Gata3) (Kaufman et al., 2003), whose expression is expected to be downregulated in bulge stem cells, showed a decreased expression in both α6+/MTS24+ and α6+/CD34+ keratinocytes. In general, we noticed that genes were more enriched within α6+/CD34+ compared to α6+/MTS24+ keratinocytes. One exception was Tnc, which encodes an extracellular matrix protein, whose expression was much higher in α6+/MTS24+ compared to α6+/CD34+ keratinocytes. This observation was supported by immunohistochemistry (data not shown). As expected, CD34 mRNA expression was nearly 20-fold lower in α6+/MTS24+ keratinocytes compared to α6+/CD34+. This finding validates our earlier observations that the MTS24 and CD34 subpopulation are distinct cell populations within the hair follicle.

**DISCUSSION**

We describe a novel subpopulation of murine follicular keratinocytes that are immunoreactive for the cell-surface marker MTS24. MTS24 is a particularly intriguing marker based upon studies in thymic epithelium, where ectopic transplantation of a small number of MTS24-positive thymic epithelial cells can give rise to a complete, functional thymus, indicating that the MTS24-positive fraction harbours epithelial stem cells (Gill et al., 2002). Our data show that
MTS24-positive follicular keratinocytes are highly clonogenic in vitro and have a gene expression pattern resembling that of bulge-derived epidermal stem cells. The high proliferative capacity, stem-like expression profile and localisation in a well-protected niche indicate that MTS24-positive keratinocytes represent a new progenitor cell located within murine hair follicles.

We have shown by immunolabelling, colony-forming assays and gene expression analysis that MTS24 and CD34 identify two distinct populations of follicular keratinocytes that exhibit divergent in vivo characteristics and in vitro function. The most thoroughly characterised epidermal stem cell population resides in the bulge region of the hair follicle outer root sheath (Cotsarelis et al., 1990; Morris and Potten, 1994; Lavker and Sun, 2000). Label-retaining cells (LRCs) are concentrated in the bulge, and these cells express the markers CD34 and keratin 15 (Trempus et al., 2003; Morris et al., 2004). Bulge cells are capable of giving rise to all the differentiated lineages of the IFE, hair follicle and sebaceous gland (Taylor et al., 2000; Oshima et al., 2001; Morris et al., 2004). The in vivo observation that MTS24 cells are infrequently label-retaining when compared to adjacent CD34-expressing cells is reminiscent of the hematopoietic stem cell system where distinct populations of short- and long-term progenitors have been described. As the multipotent, long-term reconstituting hematopoietic stem cells (LT-HSC) differentiate, the self-renewal potential of their progeny declines. Short-term hematopoietic stem cells (ST-HSC) are multipotent and renew for 6-8 weeks; these further differentiate to give rise to multipotent progenitors and finally oligolineage-restricted progenitor cells (Shizuru et al., 2005). It seems likely that a similar hierarchical restriction of lineage and self-renewal potential may exist in progenitor cells of the epidermis. In this context, we hypothesise that MTS24-positive keratinocytes may represent a short-term repopulating epidermal progenitor derived from the adjacent long-term, label-retaining CD34-expressing bulge cell population (see Fig. 7).

Despite evidence that MTS24-positive cells likely represent a short-term repopulating subset of epidermal progenitor cells, it remains entirely possible that these cells represent a bulge-independent population of stem or stem-like cells that maintain a unique regenerative capacity. Several observations generated in the course of this study support this alternative role for MTS24-positive epidermal keratinocytes. Firstly, while MTS24-reactive keratinocytes were already observed at E17 during hair follicle development in wild-type mice, CD34 expression was not detected until day 6 after birth (Fig. 2A-C). At this stage, hair follicles are almost fully developed (Schmidt-Ullrich and Paus, 2005). The initiation of MTS24 labelling in anagen follicles during both the early stages of hair follicle development and transgene-mediated ectopic hair follicle formation suggests that MTS24-positive keratinocytes are independently involved in hair follicle formation. Additionally, it has been suggested that LRCs located in the hair follicle bulge are not synonymous with epidermal stem cells, but
probably represent only a subset of the total epidermal stem cell population (Braun et al., 2003; Claudinot et al., 2005). In support of this, long-term lineage marking has shown that in undamaged epidermis there are distinct stem cell populations within the interfollicular epidermis, sebaceous glands and hair follicles (Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002). Furthermore, permanent in vivo lineage-tagging experiments in transgenic mice have shown that bulge cells are not responsible for normal maintenance of the interfollicular epidermis (Ito et al., 2005; Levy et al., 2005), and ablation of keratin 15-positive cells results in a complete loss of the bulge microenvironment with no effect on the interfollicular epidermis (Ito et al., 2005). As we have demonstrated that MTS24-positive keratinocytes are distinct from keratin 15-positive cells, it remains possible that these cells survive transgene-mediated ablation and contribute to prolonged interfollicular epidermal survival.

The relationship between the MTS24+ and CD34+ progenitor cell subpopulations remains to be clarified. We propose three potential models (Fig. 7). First, MTS24+ keratinocytes may represent a population of committed progenitor cells that are derived from the CD34+ bulge stem cells, analogous to the restricted-lineage progenitor populations in the hematopoietic system. The second model suggests that the MTS24 population may represent a subset of hair follicle stem cells that have adapted their cell-surface marker repertoire to the local microenvironment. Interactions with the surrounding niche probably regulate stem cell migration, proliferation and lineage specification (Fuchs et al., 2004). The final model is that MTS24+ cells represent a follicular stem cell population that is completely autonomous from the CD34+ population of the follicular bulge.

To begin to determine which of these three models best describes the MTS24+ basal keratinocytes, we compared the colony-forming efficiency in culture and gene expression profile of MTS24+ and CD34+ basal keratinocytes. Our data show that α6+/CD34+ bulge stem cells were approximately twice as efficient as α6+/MTS24+ keratinocytes at forming large colonies in culture. However, both α6+/CD34+ and α6+/MTS24+ keratinocytes showed increased colony-forming efficiency in comparison with the unfractonated ‘all sorted’ population. Furthermore, both α6+/CD34+ and α6+/MTS24+ keratinocytes generated large colonies containing many small, undifferentiated keratinocytes, and passed efficiently to form secondary colonies of equivalent size to each other, which provides evidence for the stem-like nature of the two subpopulations. We analysed the self-renewal capacity of purified keratinocytes in vitro because it has been reported that clonogenic keratinocytes are closely related to the multipotent epidermal stem cells (Kobayashi et al., 1993; Rochat et al., 1994; Oshima et al., 2001). The results of these primary and passaged cell assays indicated an enhanced colony-forming efficiency of MTS24-positive cells which was comparable to established, CD34 positive bulge-associated HF stem cells. While these methods do verify that MTS24-positive cells are epidermal progenitors, they do not necessarily allow us to examine the differentiation potential of these cells along sebaceous or hair-follicle lineage pathways. Future studies using ex vivo cell engraftment will address the lineage commitment of MTS24-positive epidermal progenitors.

Our Q-PCR results confirm that CD34 is not significantly enriched in the α6+/MTS24+ sorted keratinocytes, providing convincing evidence that these subsets of cells are non-overlapping. Both α6+/CD34+ and α6+/MTS24+ keratinocytes showed a similar gene expression profile for genes that were expected to be up- or downregulated in hair follicle stem cells compared to non-stem cells (Morris et al., 2004; Tumbar et al., 2004; Claudinot et al., 2005). However, in general, gene expression was more enriched in α6+/CD34+ compared to α6+/MTS24+ keratinocytes. Future studies will seek to address the effect of individual genes on the function of the bulge and MTS24-positive cells. Taken together, the colony formation and genetic profiling data appear to support our first model that MTS24+ keratinocytes represent a population of committed progenitor cells that are derived from the CD34+ bulge stem cells.

The role of MTS24-positive keratinocytes in the hair follicle remains to be analysed. In the thymus, MTS24 was reported to identify epithelial progenitor cells that not only function to reconstitute a full epithelial compartment of the thymus but were also able to create functional microenvironments supporting normal T cell development (Bennett et al., 2002; Gill et al., 2002). We hypothesise that the reservoir of MTS24-positive hair follicle keratinocytes could have similar properties; i.e. MTS24-positive keratinocytes could play an important role in organising a cellular microenvironment required for epidermal homeostasis. Our observation that MTS24 labelling was already found in the early stages of embryonic hair follicle development supports this hypothesis. The location of MTS24-positive keratinocytes in a sequestered microenvironment adjacent to the bulge and isolated from the changes that occur in the hair follicle as it cycles, suggests that these cells are biologically important. MTS24-positive keratinocytes appear to be ‘well-placed’ to produce progeny to replenish the interfollicular epidermis, sebaceous gland and/or hair follicle lineages. To assess the lineage potential of MTS24+ keratinocytes, it will be necessary to purify these cells and to assess their ability to contribute to epidermal skin grafts or, preferably, to use permanent in vivo lineage marking to assess the fate of these cells in intact epidermis. Future work will seek to clarify the origin and role of MTS24-positive keratinocytes during normal homeostasis and in conditions such as skin wounding and following transplantation.

In summary, our findings demonstrate that the membrane-bound marker MTS24 selects for a novel population of follicular keratinocytes with an undifferentiated phenotype, high proliferative potential and a gene expression pattern resembling that of follicular stem cells. We have shown that MTS24 labelling is found in the early stages of hair follicle development and during de novo hair follicle formation. Future experiments will seek to determine whether the MTS24-positive keratinocytes represent a new reservoir of epidermal stem cells or a population of lineage-restricted progenitor cells. Either outcome would be interesting since markers for progenitor keratinocytes have yet to be identified within the hair follicle. Furthermore, characterisation of the molecular and functional attributes of MTS24-positive epidermal cells may provide targets for modifying keratinocyte progenitor cell behaviour in circumstances such as alopecia, wound healing and cancer. Finally, MTS24 has now been reported as a putative marker of both thymic and epidermal progenitor cells. Therefore, elucidation of the functional properties of the MTS24 cell-surface antigen will probably provide broad insights regarding progenitor cell biology of multiple epithelial organs.

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Supplemental material

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