Markers to Evaluate the Quality and Self-Renewing Potential of Engineered Human Skin Substitutes *In Vitro* and after Transplantation

Luca Pontiggia[1], Thomas Biedermann[1], Martin Meuli[1], Daniel Widmer[1], Sophie Böttcher-Haberzeth[1], Clemens Schiestl[1], Jörg Schneider[1], Erik Braziulis[1], Irene Montaño[1], Claudia Meuli-Simmen[1,2] and Ernst Reichmann[1]

We screened a series of antibodies for their exclusive binding to the human hair follicle bulge. In a second step these antibodies were to be used to identify basal keratinocytes and potential epithelial stem cells in the human epidermis and in engineered skin substitutes. Of all the antibodies screened, we identified only one, designated C8/144B, that exclusively recognized the hair follicle bulge. However, C8/144B-binding cells were never detected in the human epidermal stratum basale. In the bulge C8/144B-binding cells gave rise to cytokeratin 19-positive cells, which were also tracked in the outer root sheath between bulge and the hair follicle matrix. Remarkably, cytokeratin 19-expressing cells were never detected in the hair follicle infundibulum. Yet, cytokeratin 19-expressing keratinocytes were found in the epidermal stratum basale of normal skin as a subpopulation of cytokeratin 15-positive (not C8/144B-positive) basal keratinocytes. Cytokeratin 19/cytokeratin 15-positive keratinocytes decreased significantly with age. We suggest that cytokeratin 19-expressing cells represent a subpopulation of basal keratinocytes in neonates and young children (up to 1.5 years) that is particularly adapted to the lateral expansion of growing skin. Our data show that cytokeratin 19 in combination with cytokeratin 15 is an important marker to routinely monitor epidermal homeostasis and (at least indirectly) the self-renewing potential of engineered skin.

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INTRODUCTION

Epidermal self-renewal in native skin indispensably requires adult epidermal stem cells (SCs). This is equally true for all sorts of surgically harvested as well as laboratory grown skin grafts. Epidermal SCs are thought to reside in the basal layer of the interfollicular epidermis and are supposed to be unipotent SCs that give rise to only one cell lineage, namely keratinocytes (Brouard and Barrandon, 2003). Furthermore, there is convincing evidence that multipotent epithelial SCs are located in the bulge region of the hair follicle (HF; Blanpain *et al.*, 2004; Morris *et al.*, 2004). These cells have the potential of generating at least three different cell lineages including hair matrix cells, sebaceous gland cells, and epidermal keratinocytes (Alonso and Fuchs, 2003).

[1]*Tissue Biology Research Unit, Department of Surgery, University Children's Hospital, Zurich, Switzerland and* [2]*Klinik für Plastische u. Wiederherstellungschirurgie, Kantonsspital, Tellstrasse, 5001 Aarau, Switzerland*

Correspondence: PD, Dr. Ernst Reichmann, Tissue Biology Research Unit, Department of Surgery, University Children's Hospital, Steinwiesstrasse 75, Zurich CH-8032, Switzerland.
E-mail: ernst.reichmann@kispi.uzh.ch

Abbreviations: HF, hair follicle; K, cytokeratin; PBS, phosphate-buffered saline; SC, stem cell

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Although significant advances were made in identifying and locating the epithelial stem cell compartment in rodent skin (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000; Oshima *et al.*, 2001; Braun *et al.*, 2003; Blanpain *et al.*, 2004; Fuchs *et al.*, 2004; Morris *et al.*, 2004), stem cell data regarding human skin are still vague. A number of putative stem cell markers, such as β1-integrin (Jones and Watt, 1993; Jones *et al.*, 1995), α6-integrin in combination with CD71 (Li *et al.*, 1998; Webb *et al.*, 2004), CD34 (Trempus *et al.*, 2003; Blanpain *et al.*, 2004), AC133 (Belicchi *et al.*, 2004), p63 (Pellegrini *et al.*, 2001; Koster and Roop, 2004), keratin (K)15 (Lyle *et al.*, 1998), ABCG2 (Terunuma *et al.*, 2003; Triel *et al.*, 2004), and BMI-1 (Park *et al.*, 2003) have been suggested. However, convincing evidence that these markers exclusively identify cells that have the capacity to self-renew and to maintain long-term tissue integrity and function is mostly missing.

Clinical follow-up studies demonstrate that epidermis generated from cultured epidermal autografts can self-renew for decades (Carsin *et al.*, 2000), indicating that a sufficient number of viable stem cells was initially present and survived in the transplanted graft. On the other hand there is also evidence that freshly transplanted cultured epidermal autografts are lost, due to the melting graft phenomenon, when grafts contain an insufficient number of viable epidermal stem cells (Matsumura *et al.*, 1998).

According to the literature there are different views on how keratinocyte stem cells are organized in human skin. They may be arranged as epidermal proliferative units (Mackenzie, 1970; Potten and Morris, 1988), or be derived from skin appendages. Also, homeostatic regulation may cause suprabasal (K10 expressing) keratinocytes to retro-differentiate into unipotent self-renewing keratinocytes (Li *et al.*, 2004), for instance in a wound with a shortage of self-renewing keratinocytes.

On the basis of the above considerations we aimed at searching for tools suited to evaluate the quality and self-renewing potential of engineered human dermoepidermal skin grafts. We present data showing that the C8/144B antibody is the only one in our antibody screening that recognizes a distinct cell population in the HF bulge. This cell population gives rise to K19-positive cells which may produce matrix cells that contribute to hair growth. We also provide evidence that K19-expressing cells represent a subpopulation of keratinocytes in the human stratum basale during proliferative lateral skin expansion. We have developed a one-step dermoepidermal transplantation model which reveals that K19 is a valuable marker to monitor the quality, homeostasis, and self-renewing capacity of engineered skin substitutes. Notably, we found that the situation in human skin differs in many aspects from that in mouse.

RESULTS

Two types of epithelial cells can be distinguished in the hair follicle bulge of normal human skin

We have analyzed skin biopsies from human individuals (aged 1 day to 49 years). We intended to identify antibodies specifically binding to the human HF bulge, a region known to contain multipotent skin epithelial stem cells (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000; Oshima *et al.*, 2001; Braun *et al.*, 2003; Fuchs *et al.*, 2004). In a second step we employed selected bulge-specific antibodies to search for keratinocyte stem cells within the stratum basale. Antibodies directed to the following antigens were tested: β1-integrin, α6-integrin, CD71, ABCG2, p63, Ki67, K15, antigen detected by C8/144B, K19, CD34, melanoma chondroitin sulfate proteoglycan, PLZF, BMI-1, CD200, follistatin, Dkk3, and Wif-1 (Table 1).

The only antibody, which exclusively bound to the HF bulge, was clone C8/144B from Dako (Baar, Switzerland; Figure 1a; Table 1). The C8/144B monoclonal antibody was originally generated against a short intracytoplasmic peptide of CD8. There are reports claiming that C8/144B recognizes K15 in the bulge region of human HFs (Lyle *et al.*, 1998). Using confocal microscopy we confirmed that C8/144B indeed specifically binds to the HF bulge (Figure 1b and c; Table 1). Thus, we consider C8/144B the most reliable human HF bulge marker presently available.

The human HF bulge was also recognized by an antibody to K19, however, K19-positive cells were also detected along the variable region of the HF down to the HF matrix (Figure 1a). Importantly, K19-positive cells were never seen in the infundibulum above the bulge (Figure 1a), suggesting that HF-derived, K19-expressing cells do usually not give rise to epidermal cells (under nontraumatic conditions).

K19-positive cells originate from CD8/144B-expressing cells in the hair follicle bulge of normal human skin

The data shown in Figure 1a raise the possibility that K19-positive cells originate in the HF bulge and migrate from there

Table 1. Presumptive keratinocyte stem cell-specific antibodies

			Binding in human HF		Binding in human epidermis	
Antigen	**Reference**	**Antibody (clone)**	**Bulge**	**ORS/IRS**	**Basal layer**	**Suprabasal**
CD34	Blanpain *et al.* (2004)	581, My10, 8G12	+	+	–	–
CK19	Michel *et al.* (1996)	RCK108	+	+	+	–
CK15	Ohyama *et al.* (2006)	LHK15, SPM190	+	+	+	–
CK15	Lyle *et al.* (1998)	C8/144B	+	–	–	–
p63	Koster and Roop (2004)	4A4	+	+	+	+
Dkk3	Ohyama *et al.* (2006)	Polyclonal	–	–	–	–
Wif-1	Ohyama *et al.* (2006)	Polyclonal	–	–	–	–
MCSP	Legg *et al.* (2003)	LHM-2	+	+	+	–
CD133	Belicchi *et al.* (2004)	AC133	+	+	+	–
Follistatin	Ohyama *et al.* (2006)	85918	+	+	+	–
β1-Integrin	Jones and Watt (1993)	7F10	+	–	+	+
PLZF	Costoya *et al.* (2004)	2A9	+	+	–	+
BMI-1	Park *et al.* (2003)	22F6	+	+	+	+
ABCG2	Triel *et al.* (2004)	5DS	–	+	+	+

ORS/IRS, outer root sheath/inner root sheath
The presumptive keratinocyte stem cell-specific antibodies, tested on interfollicular epidermis and the hair follicle outer root sheath.

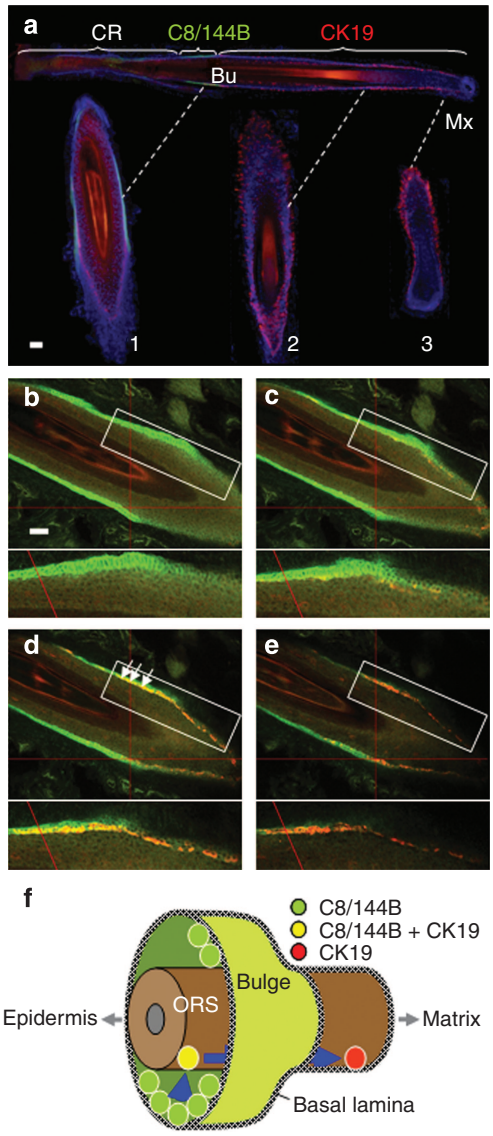

Figure 1. Two different cell types can be distinguished in the human hair follicle bulge. (**a**) Double immunofluorescence employing C8/144B (green) and anti-K19 (red) antibodies on scalp skin of a 3-year-old child. The positions of the three cross-sections through the hair follicle are indicated by 1, 2, and 3. C8/144B exclusively binds to cells of the HF bulge (Bu and 1). K19-specific antibodies recognize cells in the bulge and all along the outer root sheath (2) down to the HF matrix (Mx and 3). Nuclei are counterstained with the Hoechst dye 33342 (blue). None of the two antibodies binds to the constant region (CR) above the HF bulge. (**b**–**e**) Serial confocal sections show the asymmetric organization of the HF bulge and reveal C8/144B-binding multipotent stem cells (green). Boxes (white frames) indicate the magnified region depicted underneath. (**c**, **d**) A thin layer of C8/144B and K19-double-positive cells (arrows in **d**, and yellow cells in the magnified field in **d**) becomes obvious underneath the layer of C8/144B-positive cells. (**e**) Bulge-derived, K19-expressing cells at the lower end of the bulge region. (**f**) Scheme, summarizing the immunofluorescence patterns (Bu, bulge; CR, constant region; Mx, hair follicle matrix). All scale bars: 50 μm.

to the HF matrix. Therefore, we also performed double immunofluorescence using C8/144B and K19 antibodies and confocal microscopy on HF bulge sections (Figure 1c–e).

We identified a region in the asymmetrically organized bulge, which consisted almost exclusively of cells recognized by the C8/144B antibody (Figure 1b). As the optical sections approached the region underneath the C8/144B-positive cells (toward the centrally located hair shaft), a short zone of cells was detected by both the C8/144B (FITC conjugated) and the anti-K19 (PE conjugated) antibodies resulting in a yellow fluorescence (Figure 1c and d). Towards the inner cell layers of the bulge, cells recognized by C8/144B became rare, whereas K19-expressing cells were more abundant (Figure 1e). These data imply that C8/144B-binding and K19-expressing cells are organized in distinct layers in the human HF bulge. The existence of a small transitional zone in which both markers are expressed in the same cells suggests that K19-positive cells are directly derived from C8/144B-binding cells.

A scheme summarizing these results is shown in Figure 1f. In accordance to other published data, our findings suggest that there are two distinct populations of epithelial cells in the human HF bulge (Blanpain *et al.*, 2004). However, in contrast to Blanpain *et al.*, who described a CD34-positive cell population restricted to the mouse HF bulge, we detected CD34-expressing cells all along the outer root sheath in the human HF.

C8/144B does not recognize interfollicular keratinocytes

Upon testing the C8/144B antibody no cells were recognized in human interfollicular epidermis. This was invariably the case in human skin derived from different sites, such as foreskin, scalp, abdomen, back, and retroauricular (Table 2). This finding suggests that (multipotent) stem cells in the HF bulge are distinct from (unipotent) stem cells located in the epidermis.

To gain more insight into their K15 specificity, we compared C8/144B with two commercially available, K15-specific antibodies, designated LHK15 and SPM190. Proteins were extracted from HaCaT cells, which are known to express K15 on cell culture plastic (Werner and Munz, 2000) and analyzed in western blots. The LHK15 antibody clearly recognized a band of 55 kDa (Figure 2). SPM190 also showed the same prominent band, however, also weakly detected some additional proteins. Interestingly, C8/144B also showed weak binding to the diagnostic protein (Figure 2). Differences in the affinity of the three antibodies to K15 may account for these variations in staining intensities.

K19-positive cells are a subpopulation of K15-expressing keratinocytes in the interfollicular epidermis of normal human skin

In very young skin (from neonate to 1.5-year old) K15 was expressed in all keratinocytes of the stratum basale (Figure 3a), whereas in the skin of older patients it was expressed in cells of the lower parts of the rete ridges only (Figure 3b). Suprabasal keratinocytes did not show K15 expression. These data are in accord with previous studies (Waseem *et al.*, 1999; Ghali *et al.*, 2004; Porter *et al*, 2000; Webb *et al.*, 2004) and imply that in human interfollicular epidermis K15 is not necessarily a stem cell marker but rather a marker for

Table 2. K19 and K15 expression in basal keratinocytes in correlation with donor sites and donor age

Scalp			Abdomen		
Years	CK15	CK19		CK15	CK19
1.5	++	+	8 days	+++	+++
1.8	++	–	10 months	+++	+
2.2	++	–	17 years	++	–
6.3	++	–	18 years	++	–
6.3	++	–	41 years	++	–
7.5	++	–	49 years	++	–
14	++	–			
			Back		
				CK15	CK19
			1 days	+++	+++
Retroauricular			Foreskin		
	CK15	CK19	years	CK15	CK19
21 days	+++	+++	1	+++	++
11 years	++	–	6	++	–
12 years	++	–	15	++	–

K19 expression is detectable in skin of children not older than 2 years, whereas K15 is expressed in all sites, at all ages indicated.
+, 5–10% of basal keratinocytes.
++, 20–30% of basal keratinocytes.
+++, 50–70% of basal keratinocytes.
–, no basal keratinocytes recognized.

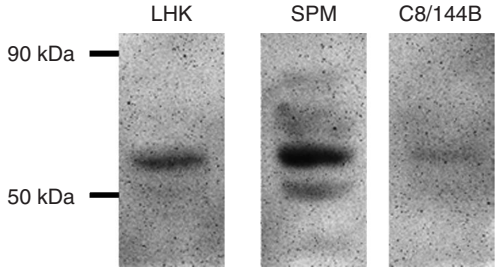

Figure 2. Testing different K15-specific antibodies by immunoblotting. Three different antibodies, LHK15, SPM190, and C8/144B were used. LHK15 and SPM190 clearly recognized a band of 55 kDa. C8/144B also showed some weak binding to the 55 kDa band. Equal volumes (25 µl) of the identical lysate were loaded.

basal keratinocytes anchored to a functional basement membrane.

K19-positive basal keratinocytes were found in all body sites investigated (Table 2). Importantly, K19 was expressed in an age-dependent manner. The number of K19-positive basal cells accounted for 50–70% of basal keratinocytes in newborn skin (Figure 3c), 10–30% in infant skin (8 days to 2 years), and was hardly detectable in adult skin (18–49 years; data not shown).

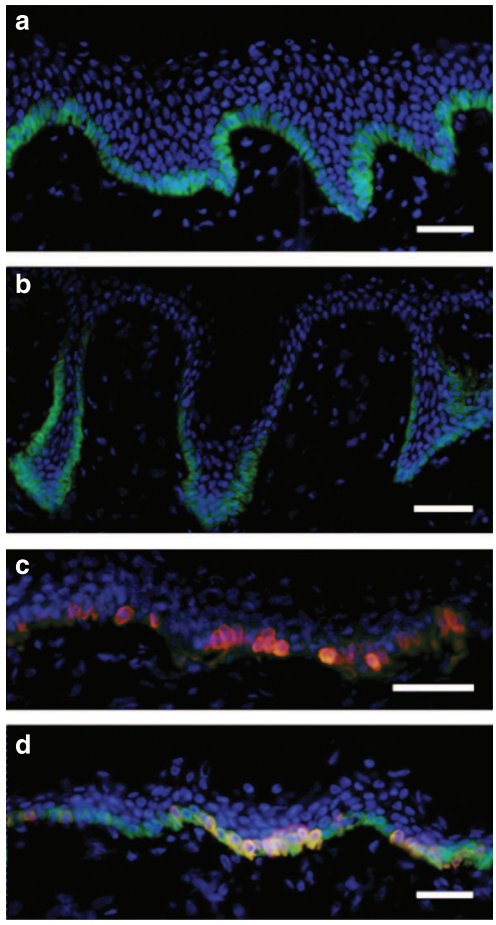

Figure 3. K19-expressing cells are a subpopulation of K15-positive keratinocytes. (**a**) All basal keratinocytes of the abdominal epidermis of a 10-month-old child express K15 (green). (**b**) Basal keratinocytes derived from the back of an 8-year-old child express K15 in the tips of the rete ridges. (**c**) About 50% of all basal keratinocytes isolated from the back of a 1-day-old neonate express K19 (red). (**d**) Double immunofluorescence using K15 (green) and K19 (red) antibodies on the epidermis derived from the retroauricular skin of a 3-week-old child. Note that K19-positive cells are a subpopulation of K15-expressing cells. Nuclei are counterstained with the Hoechst 33342 dye (blue). All scale bars: 50 µm.

K19-positive cells represented a subpopulation of K15-expressing basal keratinocytes (Figure 3d). K19/K15-expressing basal keratinocytes were almost always arranged in clusters (Figure 3d). K20 staining demonstrated that about 0.1% of all K19-expressing cells were Merkel cells (data not shown).

Basal keratinocytes-expressing K19 are indicators of an intact epidermal homeostasis in dermoepidermal substitutes engineered *in vitro*

To evaluate the fate of K19-expressing cells on a plastic substrate in culture, epidermal keratinocytes derived from children and adults (Table 2) were immunostained with K19 antibodies. At 2 days after plating, keratinocytes were completely spread out on the substrate. Several K19-positive cells were arranged in pairs (Figure 4a) indicating that the first mitoses had occurred. After 4 days in culture, K19-positive

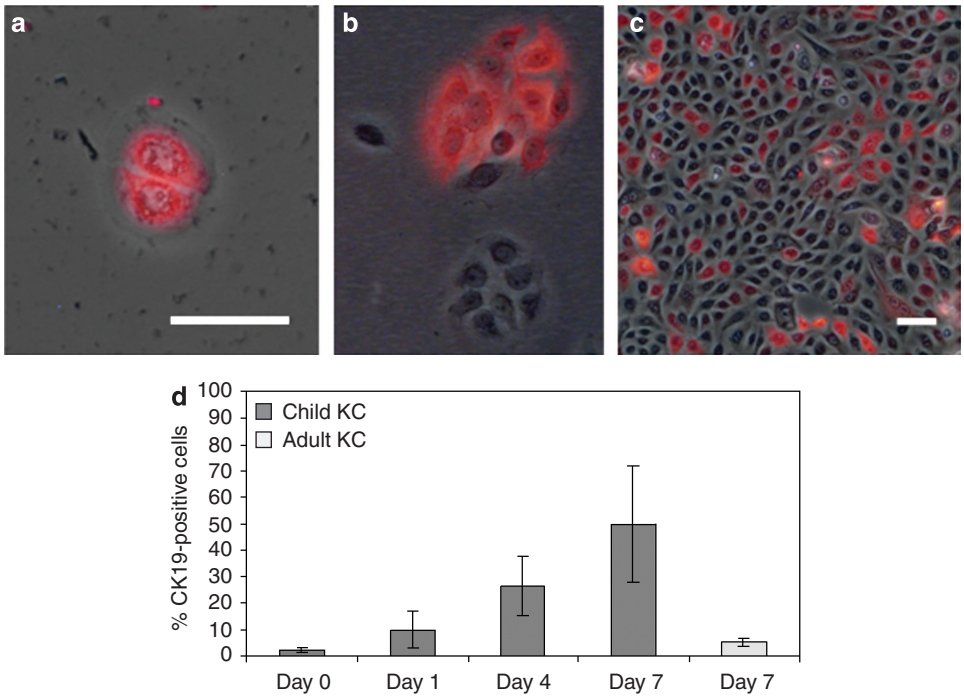

Figure 4. K19-positive keratinocytes proliferate under non-homeostatic conditions on cell culture plastic. (**a**) Doublets of K19-expressing keratinocytes. (**b**) K19-positive and K19-negative colonies of human keratinocytes can be observed 4 days after plating. (**c**) A confluent layer of keratinocytes 8 days after plating. A total of 30–50% of the cells express K19. (**d**) Quantification of K19-expressing cells isolated from children (from neonate to 5 years) at four different time points, reveals a steadily increasing number of K19-positive keratinocytes on cell culture plastic. Notably, adult (40 to 50-year old) keratinocytes show 10 times less K19-positive cells after 7 days in culture. Dark columns represent results with infant keratinocytes (infant KC). Bright columns represent results with adult keratinocytes (adult KC).

cells were still arranged in colonies (Figure 4b). After 7–10 days, the keratinocytes had become confluent in culture and about 30–50% of the cells expressed K19 (Figure 4c). The significant increase of K19-expressing keratinocytes derived from young children (up to 5 years) is shown in Figure 4d. In contrast, adult keratinocytes never gave rise to this high numbers of K19-expressing cells. In keratinocyte cultures derived from 40 to 50 years old patients we found 3–5% of K19-posive cells 7, 9, and 15 days after plating (Figure 4d). These data suggest that the *in vitro* outgrowth of K19-positive keratinocytes decrease with increasing age. They also underscore the value of K19 expression as a marker to monitor the self-renewing and regenerating potential of a given epidermal substitute.

To determine the epidermal regenerative capacity of keratinocytes, primary cells were grown into multilayered epidermal constructs. Keratinocytes were plated onto collagen type-I hydrogels populated by dermal fibroblasts on which they developed into multilayered epidermal equivalents within 3 weeks (Figure 5a). These exhibited a basal layer of densely packed cells, followed by 10–15 layers of differentiating keratinocytes and some layers of terminally differentiated cells forming a stratum corneum (Figure 5a). Upon organotypic dermoepidermal culture on collagen type-I hydrogels, homeostatic regulation caused excess K19-positive keratinocytes (previously expanded on plastic) to be eliminated by their release into terminally differentiated strata. However, a substantial number of K19-expressing cells remained in the basal layer (Figure 5b). A basal lamina-like structure had been deposited, as demonstrated by antibodies to laminin 10 (Figure 5c). Furthermore, K15 was not expressed in the basal cells of these constructs (data not shown), indicating that the *in vitro* grown epidermis still lacks some significant organizational properties *in vitro*. However, as these substitutes perfectly survived after transplantation onto immunoincompetent nude rats, it is clear that (although K15 was not expressed in these basal keratinocytes) a self-renewing keratinocyte compartment was existent. Ki67-expressing, that is proliferating keratinocytes, were present 4 weeks after seeding and were located almost exclusively basally (Figure 5d). In contrast to the situation on cell culture plastic, only a small percentage (3–5%) of K19-expressing cells was positive for Ki67, indicating cell proliferation (see also Figure 6h).

These findings are consistent with the view that K19-positive keratinocytes in the stratum basale reveal a ''young'' epidermis, and are indicators of intact tissue homeostasis in engineered skin substitutes.

Basal K15/K19-expressing cells are indicators of epidermal homeostasis in skin resulting from grafted *in vitro* engineered dermoepidermal substitutes

We sought to determine the regenerative potential and homeostasis of dermoepidermal composites after transplantation. Full thickness skin defects were created on immunoincompetent Nu/Nu rats and sheltered against the surrounding

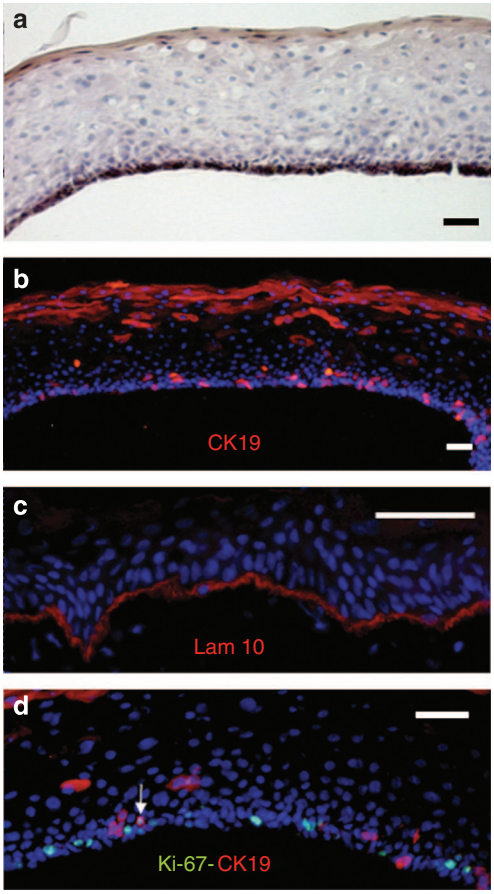

Figure 5. Evaluation of engineered epidermis equivalents. (**a**) Histological section and H/E staining of an *in vitro* engineered human epidermis equivalent grown on a collagen type-I gel which contains human dermal fibroblasts, 4 weeks after plating. The epidermis equivalent consists of a stratum basale, 10–15 keratinocyte layers, and a stratum corneum. (**b**) K19-expressing keratinocytes are apparently not yet polarized and are somewhat scattered in the basal layer. Homeostatic regulation in an organotypic graft causes excess K19-positive keratinocytes (created during their propagation on cell culture plastic) to be terminally differentiated, and hence eliminated, during stratification. (**c**) Laminin 10 staining shows that a basement membrane is about to be deposited. (**d**) Ki67/K19 double staining. Proliferating, Ki67-expressing, keratinocytes are located basally. The arrow indicates a rare Ki67/K19-double-positive keratinocyte. All scale bars: 50 μm.

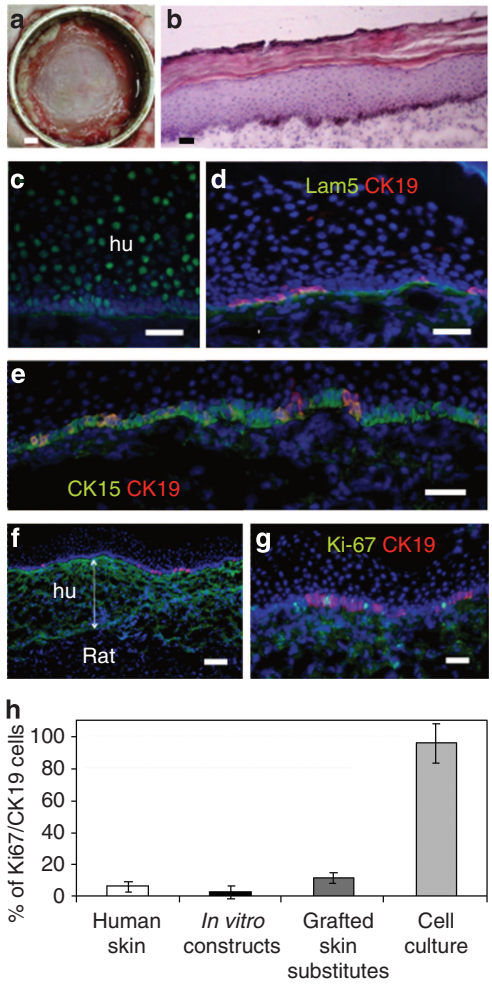

Figure 6. K19-positive human keratinocytes are indicative of an intact epidermal homeostasis in a grafted dermoepidermal skin substitute. (**a**) Dermoepidermal skin substitutes consisting of an epidermal equivalent and a collagen type-I hydrogel containing dermal fibroblasts was transplanted on the back of immunoincompetent Nu/Nu rats. At 3 weeks after transplantation, an intact human epidermis covered the former full thickness wound. (**b**) H/E staining of cryosections through the grafted area 3 weeks after transplantation shows a near normal skin histology apart from missing skin appendages. The epidermis reveals an almost normal stratification with already developing rete ridges. It is tightly connected to the underlying dermis. (**c**) The antibody specific to human nuclei confirms the human origin of the epidermis (green fluorescence). (**d**) K19-positive cells (red) are organized in clusters. Note that K19-positive cells are restricted to the well-defined stratum basale. These cells are strictly adhering to the continuous basement membrane (green) which here is visualized by a laminin-5 antibody. (**e**) In contrast to the nontransplanted engineered graft, K15 (green) is now expressed in all keratinocytes of the basal-cell layer. K19-expressing cells (red) are a subpopulation of K15-positive keratinocytes. (**f**) Human dermal fibroblasts (green) are recognized by the human CD90 (Thy-1)-specific antibody, whereas the rat tissue underneath stains negatively. (**g**) Within the clusters of K19-expressing cells only few, if any, cells were proliferating as shown by antibodies to the cell-cycle marker Ki67. (**h**) The vast majority of K19-expressing keratinocytes are slowly proliferating (or nonproliferating) as seen in human skin, in engineered constructs *in vitro*, and after their transplantation *in vivo*. In contrast, when dissociated and grown on cell culture plastic, almost every K19-expressing cell becomes Ki67-positive, thus indicating intensive cell proliferation. All scale bars: 50 μm.

skin by implanting a transplantation chamber, a modified Fusenig chamber (Fusenig *et al.*, 1983), to prevent wound healing through rat-derived cells (Figure 6a).

The only dermoepidermal skin grafts that were rapidly and sufficiently vascularized after transplantation, and hence readily integrated into the wound, were those based on collagen type-I hydrogels. Importantly, these grafts could be transplanted in only one single surgical intervention. Histological analyses 21 days after transplantation revealed an epidermis with a near to normal stratification and discernable rete ridges (Figure 6b). The human origin of the transplanted keratinocytes was confirmed using a mouse anti-human nuclei monoclonal antibody (Figure 6c). K19-positive cells were relatively abundant and formed monolayered clusters, firmly attached to a basement membrane-like

structure, as demonstrated by K19/laminin 5 double staining (Figure 6d). The number of K19-expressing cells, 3 weeks after transplantation was comparable to the number found in the skin of 0–2 years old children. Importantly, this was also true for grafts derived from keratinocytes of adult donors.

In contrast to nontransplanted skin substitutes, transplanted grafts exhibited a continuous layer of basal cells expressing K15, with K19-positive cells representing a subpopulation of these (Figure 6e). This is identical to the situation in native skin (Figure 3d).

Obviously, human dermal fibroblasts, initially submerged in polymerizing collagen type-I, remained viable and proliferated after transplantation, as a near normal dermal layer of human origin was clearly distinguishable from the underlying rat tissue using an antibody recognizing human fibroblasts, 3 weeks after transplantation (Figure 6f). Thus, dermoepidermal grafts after transplantation matured into tissues closely resembling normal human skin.

Co-staining the stratum basale with Ki67 and K19-specific antibodies and the subsequent quantification of double-positive cells revealed that in human skin, in engineered grafts (*in vitro*), and in transplanted engineered substitutes (Figure 6g), the number of double-positive cells was about 5% of all K19-positive keratinocytes (Figure 6h). These findings are in accord with the hypothesis that the vast majority of K19-expressing keratinocytes are slowly proliferating (or nonproliferating). In contrast, 90% of the K19-expressing keratinocytes derived from young children (up to 5 years), when grown on cell culture plastic, became Ki67-positive and hence proliferating (Figure 6h). As a consequence these ''young'' (and initially quiescent) K19-positive cells were finally dominating the culture.

Comparing K19 expression with other potential keratinocyte stem cell markers

We were wondering whether K19-positive keratinocytes were also recognized by other described keratinocyte stem cell markers. In particular the combination of α6-integrin and CD71 (transferrin receptor) antibodies appears to be relevant in this respect. α6-Integrin-bright (bri)/CD71-dim keratinocytes have been published to be enriched for keratinocyte stem cells (Li *et al.*, 1998; Tani *et al.*, 2000). Our Fluorescence activated cell sorting (FACS) analyses revealed that the α6-integrin-bri/CD71-dim fraction shows indeed significantly more K19-positive keratinocytes (up to 3 × more) than the initially prepared, total keratinocyte population (Figure 7). The α6-integrin-dim population contained almost no K19-positive cells (data not shown).

	Total	R4 – R21 ($\alpha6^{bri}CD71^{bri}$)	R21 ($\alpha6^{bri}71^{dim}$)
CK19+	1%	21%	49%

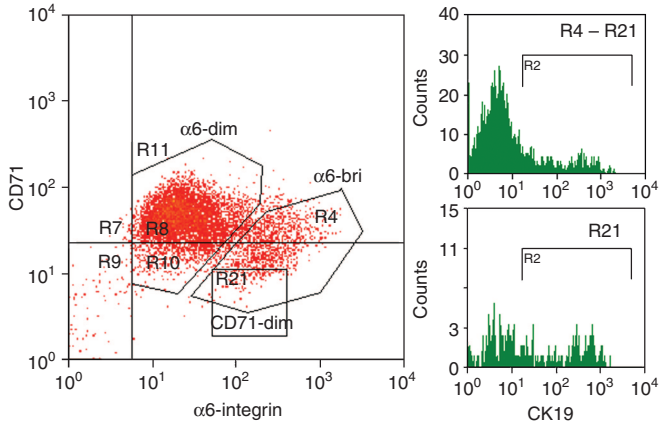

Figure 7. Comparing K19-positive keratinocytes and α6-integrin-bright/CD71-diminished cells. Staining primary keratinocytes for α6-integrin, results in two major cell populations, one of which shows low α6 expression (α6-dim), whereas the other exhibits high α6 expression (α6-bri). A subpopulation of α6-bri cells expresses low levels of CD71 (α6-bri/CD71-dim). α6-bri/CD71-dim cells are thought to contain keratinocyte stem cells. FACS analyses reveals that these α6-bri/CD71-dim fraction contains 49% K19-positive keratinocytes, whereas only 1% of the total keratinocyte preparation are K19 positive.

DISCUSSION

The goal of this study was to identify markers suited to evaluate epidermal homeostasis and the self-renewing potential of skin substitutes, both *in vitro* and after transplantation. As the C8/144B antibody binds exclusively to the HF bulge, the corresponding cells are most likely identical to multipotent epithelial stem cells (Cotsarelis, 2006). We provide evidence that these cells give rise to two distinct cell fractions. One C8/144B-binding fraction remains in place to maintain the multipotent stem cell pool. The second fraction develops into a transiently existing cell population which binds both C8/144B and K19-specific antibodies. These double-positive cells then give rise to K19-expressing cells which are no longer recognized by C8/144B. According to the stem cell migration hypothesis (Fuchs *et al.*, 2001; Oshima *et al.*, 2001), K19-positive cells may represent unipotent, self-renewing keratinocytes exiting the bulge and migrating along the outer root sheet to the basis of the HF. Here they may maintain the pool of matrix cells that contribute to hair growth.

K19-expressing keratinocytes are not found in the constant region of the HF above the bulge. Hence, bulge-derived K19-positive cells may not contribute to epidermal renewal (under normal, homeostatic conditions). This view is supported by convincing recent reports showing that bulge-derived stem cells are not responsible for maintaining the interfollicular epidermis in homeostatic conditions (Claudinot *et al.*, 2005; Ito *et al.*, 2005; Levy *et al.*, 2005).

Nevertheless, we found a distinct population of K19-positive keratinocytes in both the stratum basale of young individuals and the basal layer of engineered, stratified skin substitutes. The clustered pattern of these keratinocytes corresponds to the distribution one would expect for self-renewing keratinocytes in the stratum basale. Concomitantly, there are several reports stating that K19-expressing keratinocytes represent self-renewing cells (Lane *et al.*, 1991; Jones *et al.*, 1995; Michel *et al.*, 1996; Cotsarelis *et al.*, 1999; Akiyama *et al.*, 2000).

On the basis of our data the following questions arise: What are the properties of the distinct set of K19-positve keratinocytes in the human stratum basale? Why are K19-expressing cells so dramatically reduced with age?

K19-positive keratinocytes are highly abundant in the epidermis of fetuses and neonates (Van Muijen *et al.*, 1987; Kwaspen *et al.*, 1997). We show here that K19-positive keratinocytes are still detectable in the stratum basale of 1.5-year-old children. The epidermis of these individuals is characterized by rapid lateral growth. We suggest that K19-positive cells represent a population that is adapted to this rapid lateral expansion of the epidermis and represents a keratinocyte population that is uncommitted to terminal squamous differentiation. As with progressing age this lateral growth ceases, this type of cell is no longer required. Stratification and permanent regeneration (vertical maintenance), however, are ongoing in adult skin. This of course also requires self-renewing keratinocytes, which in adult skin are extremely rare or K19-negative. It still remains to be determined where in the adult, interfollicular self-renewing keratinocytes are derived from. As in the adult, significant numbers of K19-expressing cells are located in the HF, it cannot be excluded that these cells contribute to the basal epidermal cell pool, should there be a shortage of self-renewing cells. An additional interesting possibility is that in humans keratinocyte stem cells may derive from sweat glands (which are distributed almost throughout the entire human skin), an issue that still is only moderately investigated.

How do K19-expressing human keratinocytes compare to K19-positive keratinocytes in mouse? The murine *K19* gene shows high homology to its human counterpart, and the gene is located in the acidic keratin cluster on mouse chromosome 11 (Lussier *et al.*, 1990). In both species precursor cells in different tissues display high K19 levels (Brembeck and Rustgi, 2000). However, in mouse K19 is expressed in the HF but is absent from the interfollicular epidermis at hairy sites (Michel *et al.*, 1996). Once again this difference has to be taken into account when comparing biological phenomena in both species.

Conventional cell culture conditions provide an environment in which dissociated (single) primary keratinocytes have to newly establish their epithelial phenotype. The regulatory mechanisms of stratification and tissue homeostasis are greatly disturbed under these culture conditions. These conditions may represent a situation of extreme wound healing under which initially quiescent K19-expressing cells enter a state of proliferation. In contrast, organotypic (dermoepidermal) culture of keratinocytes, using collagen hydrogels that contain fibroblasts, induces epidermal stratification and tissue homeostasis. As a consequence surplus K19-positive cells (having accumulated during cell expansion on a plastic substrate) are now eliminated by terminal differentiation. Yet, a physiologically reasonable number of basal K19-expressing keratinocytes remains in the basal layer.

As for the quality control of engineered skin substitutes, these basal, K19-positive cells are important indicators of a young, proliferating, and self-renewing graft.

It has been reported that the C8/144B antibody recognizes K15 and defines the location of human HF stem cells in the bulge (Lyle *et al.*, 1998). However, further work demonstrated that K15 expression is not only restricted to the bulge but is also expressed in a significant stretch of the outer root sheath of the human HF and in the stratum basale of human epidermis (Porter *et al.*, 2000). We show here that the C8/144B antibody indeed weakly recognizes K15 in immunoblots, whereas it does not detect keratinocytes in the human stratum basale. A possible explanation for this discrepancy is that in human HF bulge cells, a distinct epitope of K15 is exposed, which is masked in the keratinocytes of the stratum basale. Furthermore, it is likely that there are differences in the affinities of the three K15-specific antibodies used in our immunostainings. Additional experiments are required to shed some more light on this issue.

Using collagen type-I hydrogels and a rat transplantation model we were able to achieve rapid vascularization and functional integration of complex dermoepidermal skin substitutes after one single surgical intervention. This was not possible using porous lyophilized collagen scaffolds (sponges) for epidermal reconstitution, because vascularization was too slow to keep the epidermal substitute alive.

It was also evident that transplantation of engineered dermoepidermal composites was a crucial step in completing physiological differentiation and epidermal stratification. Employing K19 and K15 as markers, it became possible to show that the organism is an extremely efficient bioreactor and a perfect regulator of organ structure and function.

In summary it can be said that K19/K15-double-positive keratinocytes represent a distinct basal-cell population in growing skin. For engineered skin substitutes, K19 and K15 in combination are valuable tools to monitor tissue homeostasis and the potential to self-renew.

MATERIALS AND METHODS

Preparation of skin specimens

Human skin samples were taken from the scalp, the abdomen, the retroauricular region, or from foreskins. Parents or patients gave their written informed consent. The medical ethical committee of the Kanton Zurich approved all described studies. Furthermore this study was conducted according to the Declaration of Helsinki Principles.

Tissues were embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and placed in dry ice. Cryosections of 6–30 μm were cut at −30 °C.

Isolation and culture of keratinocytes and fibroblasts

Skin biopsies were digested for 15–18 hours at 4 °C in 12 U ml^{-1} dispase in Hank's buffered salt solution containing 5 μg ml^{-1} gentamycin. Thereafter the epidermis and the dermis were separated using forceps. The epidermis was further digested in 1% trypsin, 5 mM EDTA for maximal 3 minutes at 37 °C. The dermal tissue was digested in 2 mg ml^{-1} collagenase for approximately 60 minutes at 37 °C. Epidermal cells were resuspended in serum-free keratinocyte medium containing 25 μg ml^{-1} bovine pituitary extract, 0.2 ng ml^{-1} EGF, and 5 μg ml^{-1} gentamycin. A total of 4×10^6 dermal cells per Ø10 cm dish were grown in DMEM supplemented with 10% fetal

calf serum, 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 μg ml^{-1} gentamycin. Collagenase was from Sigma (Buchs, Switzerland), all other compounds were from Invitrogen (Basel, Switzerland).

Organotypic cultures

Organotypic cultures were prepared using a previously established transwell system (six-well cell culture inserts with membranes of 3.0 μm pore size; BD Falcon, Basel, Switzerland). The membranes were covered with collagen type-I hydrogels which contained 1×10^5 human dermal fibroblasts (passage 1). These dermal equivalents were grown in DMEM for 6 days to allow for gel contraction. Subsequently 5×10^5 basal keratinocytes were seeded on each dermal equivalent. Triplicate wells were set up for each dermoepidermal substitute.

Keratinocytes were cultured for 4 days in three parts of DMEM and one part of Ham's F12, 0.3% fetal calf serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 5 μg ml^{-1} gentamycin (all; Invitrogen), 0.4 μg ml^{-1} hydrocortisone, 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 2 nM triiodothyronine, 180 μM adenine, 5.3 pM sodium selenite, 20 nM progesterone, and 1.8 mM $CaCl_2$ (all; Sigma). After 4 days the keratinocyte layer was raised to the air/liquid interface and cultured for 3 additional weeks. During the first week, the culture medium was a 1:1 mix of DMEM and Ham's F12 containing the supplements described above and 2% FCS (Invitrogen). The fetal calf serum was reduced to 1% during the second and third week. Cultures were finally processed for transplantation or for cryo- and paraffin sections.

Transplantation of cultured dermoepidermal composites

Dermoepidermal grafts were transplanted onto full thickness skin defects created surgically and encased by polypropylene rings, 27 mm in diameter (modified Fusenig chamber; Fusenig *et al.*, 1983). The rings were sutured on the back of 10-week-old, female athymic Nu/Nu rats. The transplants were covered with a silicon foil. After 14 days the grafts were excised in toto and processed for cryo- and paraffin sections. Anesthesia for all procedures was performed using isoflurane (Abbott AG, Baar, Switzerland).

Antibodies

CD8 (clone C8/144B, 200 μg ml^{-1}, 1:30) from Dako (Switzerland AG, Baar, Switzerland); CD71 (clone berT9, 1:10): K19 (clone RCK108, 1:100); K20 (clone K_s20.8, 1:100); K10 (clone DE-K10, 1:100) from Santa Cruz (Labforce AG, Nunningen, Switzerland); laminin 5 (clone P3H9-2, 1:100); p63 (clone 4A4, 1:100); Dkk3 (polyclonal, 1:100); Wif-1 (polyclonal, 1:100) from Chemicon (Millipore AG, Zug, Switzerland); K15 (clone LHK15, 100 μg ml^{-1}, 1:100; clone SPM190, 100 μg ml^{-1}, 1:50); human nuclei (clone 235-1, 1:50); CD49f (clone 4F10, 1:200) from R&D Systems (Abingdon, UK); ABCG2 (clone 5DS, 1:20); follistatin (clone 85918, 1:40); melanoma chondroitin sulfate proteoglycan (clone LHM-2, 1:500) from BD Pharmingen (Basel, Switzerland); Ki67 (clone B56, 1:200); CD34, PE conjugated (clone 581, 1:50) from ABD Serotec (Dusseldorf, Germany); CD200 (clone MRC OX104, 1:20) from Dianova (Hamburg, Germany); human fibroblast (clone AS02, anti-CD90/Thy-1, 1:100) from Calbiochem (VWR, Dietikon, Switzerland); PLZF (clone 2A9, 1:50) from Upstate (Millipore AG); BMI-1 (clone 22F6, 1:100) from Spring Bioscience (AMS Biotechnology, Bioggio, Switzerland). Goat anti-mouse coupled with horseradish peroxidase (polyclonal). For double immunofluorescence, some of the primary antibodies were prelabeled with Alexa 555-conjugated polyclonal goat F(ab')2 fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit; Molecular Probes/Invitrogen, Basel, Switzerland).

Immunohistochemical staining

Sections and/or cells were fixed and permeabilized in acetone for 5 minutes at −20 °C, air dried and washed 3 × in phosphate-buffered saline (PBS). Thereafter they were blocked in PBS containing 2% BSA (Sigma) for 30 minutes. Incubation with the diluted first antibody was performed in blocking buffer for 1 hour at room temperature. Slides were washed three times for 5 minutes in PBS and blocked for additional 15 minutes. The secondary antibody was added for 1 hour. Thereafter sections and/or cells were incubated for 5 minutes in PBS containing 1 μg ml^{-1} Hoechst 33342 (Sigma) and then washed twice for 5 minutes in PBS. Finally, the probes were mounted with Dako mounting solution (Dako) containing 25 mg ml^{-1} of DABCO anti-quenching agent (Sigma).

Western blotting

HaCaT cells (3×10^6) were lysed on ice in 1 ml high salt lysis buffer (1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA) containing a proteinase inhibitor cocktail (Roche Diagnostics AG, Rotkreuz, Switzerland). The lysate was centrifuged at 15,000*g* at 4 °C for 10 minutes and the pellet was solubilized in 200 μl 9 M urea, 50 mM Tris-HCl pH 7.5 for 20 minutes at room temperature. Twenty μl of 6 × Lämmli Loading buffer containing β-mercaptoethanol was added to 100 μl of the extract, and boiled for 5 minutes. Aliquots (25 μl) of the sample were loaded per lane and SDS–PAGE was performed. Semi-dry western blotting was done according to standard protocols. After blocking (blocking reagent; Roche), the membranes were incubated overnight with primary antibodies, diluted 1:100 in blocking reagent. After three washes in Tris-buffered saline Tween-20, the membranes were incubated for 1 hour with goat anti-mouse antibodies coupled with horseradish peroxidase, diluted 1:1000 in blocking reagent, followed by three washes in Tris-buffered saline Tween-20. Detection was performed using the ECL Plus kit (GE Healthcare Europe GmBH, Otelfingen, Switzerland) and a ChemiDoc-It imaging station (UVP).

Fluorescence microscopy

Fluorescence microscopy was performed using a Nikon Eclipse TE2000-U inverted microscope equipped with Hoechst, FITC, and TRITC filter sets (Nikon AG, Egg, Switzerland). For confocal imaging, the Eclipse TE2000-U was upgraded with a Nikon C1 Laser Scanning Microscope. A helium–neon laser with 543 nm excitation was used for tetramethyl rhodamine iso-thiocyanate and an argon laser with 488 nm excitation was used for FITC. With the Plan Apo × 40 c/N.A. 0.95 objective 50 optical sections with an increment of 0.5 μm were captured. The line average was set to 4. Images were processed with Photoshop 7.0 (Adobe Systems Inc., Munich, Germany).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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