Hair Follicles Guide Nerve Migration *In Vitro* and *In Vivo* in Tissue-Engineered Skin

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TO THE EDITOR

One of the major roles of skin, beyond its barrier function, is the sense of touch. As this organ is highly exposed to physical or chemical assault leading to burns, nerve regeneration is of major importance to promote sensory recovery during the healing process. We previously showed, both *in vitro* and *in vivo*, that nerve regeneration can be markedly enhanced by incorporation of laminin or Schwann cells in tissue-engineered skin (Gingras *et al.*, 2003b; Caissie *et al.*, 2006; Blais *et al.*, 2009).

However, if these strategies promote efficient recuperation of temperature and pain perception through C- and

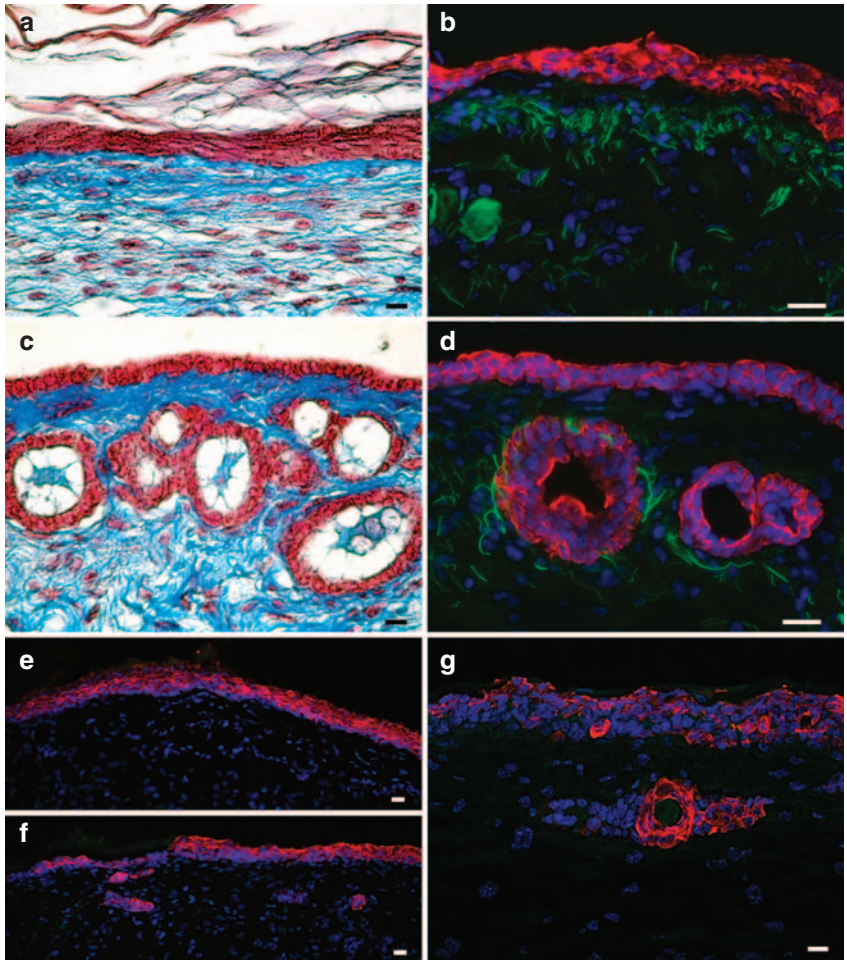

Figure 1. Hair bud-like structures guide axonal migration in the innervated tissue-engineered skin with hair bud-like structure (iTES-HBLS). In the innervated tissue-engineered skin with dissociated keratinocyte (iTES-K), a histological cross-section of tissue stained with Masson's trichrome showed keratinocytes forming a thin epidermis over the dermal sheet, which is made of mouse fibroblasts embedded in a self-assembled extracellular matrix (**a**). When hair buds were seeded on the fibroblast sheet, they formed a thin epidermis and inclusions of cells inside the upper portion of the dermis (**c**). When the iTES-K was stained by immunohistochemistry with antibodies against neurofilament M (in green) and keratin 14 (in red), neurites were observed to be randomly distributed underneath the epidermis (**b**). In the iTES-HBLS, neurites were preferentially localized around the keratin 14-positive inclusions and seemed to migrate inside the structures (**d**). The epithelial cells also expressed keratin 17 in the iTES-K (**e**) and iTES-HBLS (**f**). In a TES-HBLS (stained in red with keratin 17) without neurons, no neurofilament M-positive staining (in green) was observed, confirming that neurofilament M staining was only expressed by dorsal root ganglia neurons (**g**). Bars = 20 μm.

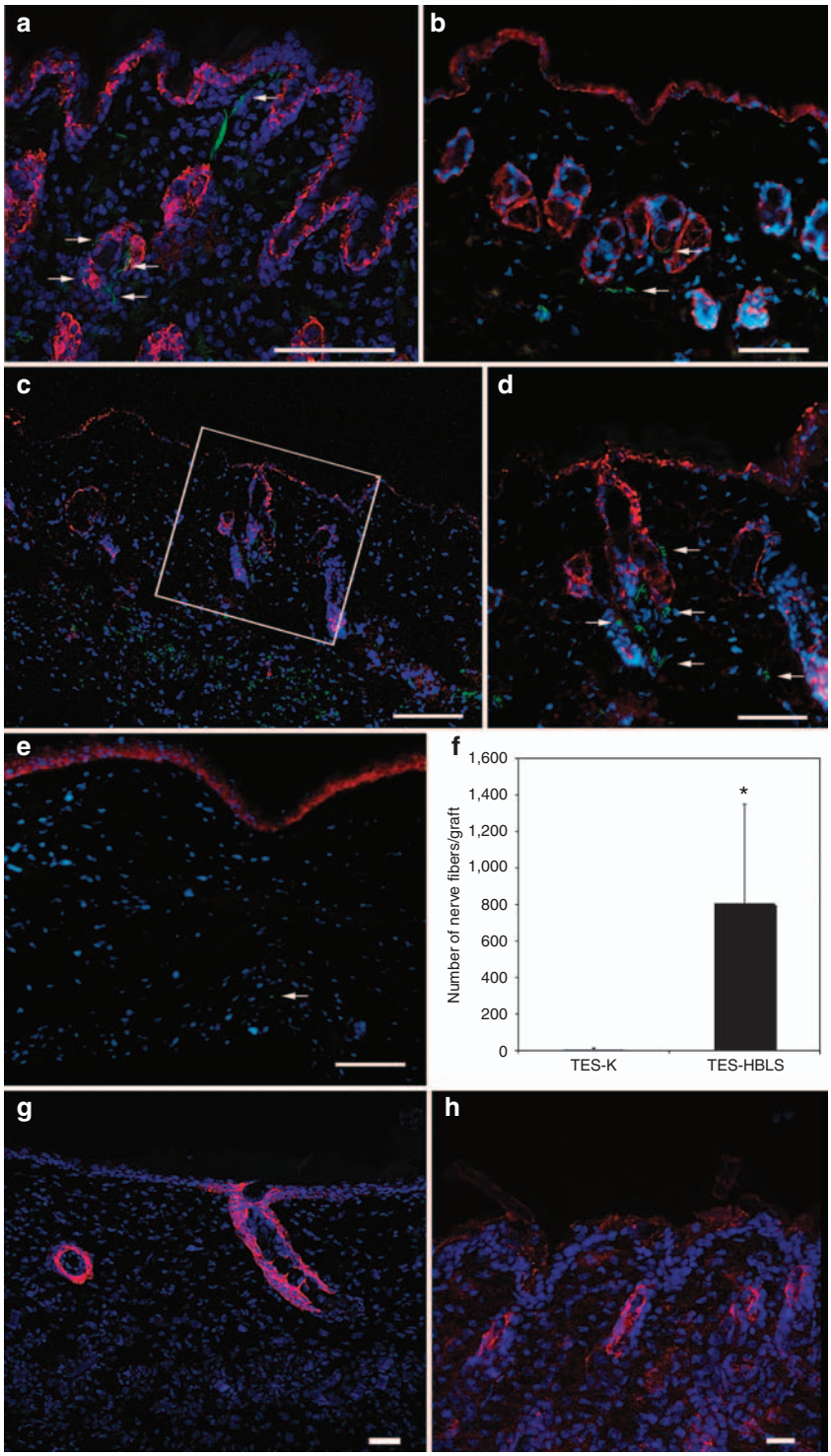

Figure 2. Hair buds sped up and guided nerve regeneration in the TES-HBLS after grafting in nude mice. At 1 month after grafting, almost no neurofilament M-positive nerve fiber (in green) was observed in the TES-K (**e**), whereas several fibers were detected, mostly in the deep dermis of the TES-HBLS (**c**). In the upper part of the dermis, most of the fibers were observed to be closely associated with keratin 14-positive hair follicles (in red, **c**; white arrows in **d**). The pattern of nerve fiber connection to hair follicles in the TES-HBLS was similar to that observed in normal black haired C3H/HeN mouse skin (white arrows in **a**) or nude mouse skin (white arrows in **b**). The number of nerve fibers detected in the TES-HBLS was significantly higher compared with the TES-K (**f**: $P<0.05$, $n=4$). In the TES-HBLS, the mature hair follicles originating from hair buds expressed keratin 17, which is a marker of epidermal appendages, and in contrast with the keratinocytes from the epidermis (**g**), as observed in normal skin of black C3H/HeN mice (**h**). Bars in **a**, **b**, **c**, and **e**: 100 μm; bars in **d**, **g**, and **h**: 50 μm.

A-delta nerve fiber regeneration, they fail to recover the sense of touch, whereas A-beta fibers successfully innervate the graft. Indeed, to restore the sense of touch, these A-beta fibers need to be connected to a sensory receptor, such as the Merkel touch dome, sensory corpuscles, or the hair follicle. Of all these sensory receptors, the hair follicle is the only one that can be used for tissue-engineering purposes.

The hair follicle constitutes a major component in the skin nerve network and is innervated by complex nerve plexuses (Botchkarev *et al.*, 1997; Peters *et al.*, 2002; Provitera *et al.*, 2007; Hendrix *et al.*, 2008). It participates in the sense of touch perception of hairy skin and, to a lesser extent, glabrous skin (Hamalainen *et al.*, 1985; Botchkarev *et al.*, 1997; Woodbury *et al.*, 2001). It has been clearly shown that hair follicles modulate skin innervation (Botchkarev *et al.*, 1997; Zhang *et al.*, 2008). Thus, we hypothesized that the incorporation of hair follicles in tissue-engineered skin may promote and/or guide nerve migration, with hairs establishing active targets for nerves (Uno and Montagna, 1982). In addition, it should greatly improve the recovery of the sense of touch, as hair follicles are sensory receptors (Woodbury *et al.*, 2001; Hendrix *et al.*, 2008).

We developed a unique model of tissue-engineered skin cultured with hair buds that grew into hairs after grafting on mice (Larouche *et al.*, 2011).

To study the influence of hair follicles on sensory neuron axonal migration *in vitro*, we prepared a dermal construct made of four superimposed self-assembled fibroblast sheets. After 1 week of maturation to promote sheet merging, the construct was seeded with mouse dorsal root ganglia sensory neurons on the top. Axonal migration was promoted throughout the three-dimensional tissue by adding nerve growth factor to the culture medium for 14 days (Gingras *et al.*, 2003a). The construct was then turned over so that the neurons were on the bottom and dissociated keratinocytes or hair buds were seeded on the top, to mimic normal cutaneous innervation and skin histology. After 1 week of culture with the construct under immersion to allow epithelial proliferation, the innervated tissue-engineered skin with hair bud-like structures (iTES-HBLSs) or with dissociated keratinocytes (iTES-Ks) was lifted up to the air–liquid interface and cultured for 2 weeks to promote epidermal differentiation.

In the iTES-K, a thin epidermis was observed covering the dermal compartment (Figure 1a). In the iTES-HBLS, a thin epidermis, made of keratinocytes originating from the hair buds, was also observed. In addition, epidermal inclusions were observed in the dermal portion, mimicking hair bud-like structures. These structures were stained with anti-keratin 14 antibodies, showing their epithelial origin (Figure 1d). In addition, when the iTES-HBLS was stained with a keratin 17-specific antibody, both HBLS and epidermis expressed the marker (Figure 1f), as well as the epidermis in the iTES-K (Figure 1e), whereas keratin 17 is a marker of epidermal appendages in normal skin (Figure 2h). However, keratin 17 is known to be expressed by hyperproliferative keratinocytes in skin substitutes cultured *in vitro* (Smiley *et al.*, 2006).

When the iTES-K was stained with antibodies against neurofilament M, numerous neurites were observed homogeneously distributed underneath the keratin 14-positive epidermis (Figure 1b). In the iTES-HBLS model, neurofilament M-positive neurites were localized preferentially around the hair bud-like inclusions and even seemed to migrate inside them (Figure 1d). As a control, no neurofilament M-positive staining was observed in a TES-HBLS (without neurons) cultured in the same conditions as the iTES-HBLS (Figure 1g).

To investigate whether these hair bud-like structures can promote normal hair growth *in vivo*, a TES-HBLS (without neurons) was transplanted on the back of a nude mouse (with approval of the Laval University animal care committee) and compared with a control TES-K. At 1 month after transplantation, a black hair tuft was observed growing from the TES-HBLS, whereas no hair was observed in the control graft with dissociated keratinocytes (Larouche *et al.*, 2011). When nerve regeneration was investigated in the grafts 1 month after transplantation, almost no neurofilament M-positive fiber was observed in the graft of the TES-K (Figure 2e). In contrast, several nerve fibers were detected deep in the dermis of the TES-HBLS graft (Figure 2c). Most of the neurofilament M-positive fibers located in the upper dermis were detected in close association with the newly formed hair follicles (white arrows, Figure 2d), in a pattern similar to that of normal C3H/HeN mouse skin (Figure 2a) or nude mouse skin (Figure 2b). The total number of nerve fibers was 180 times higher in the TES-HBLS compared with the TES-K (Figure 2f, and c vs e).

In the TES-HBLS, 1 month after grafting, keratin 17 expression was restricted to hair follicles (Figure 2g), as observed in normal C3H/HeN mouse skin (Figure 2h), and in contrast with its expression pattern before grafting (Figure 1e-g).

Thus, we showed that hair buds induced a much faster nerve migration in the graft compared with the control with keratinocytes. In addition, the nerve fibers migrating in the TES-HBLS were most often closely associated with the hair follicles in a pattern that mimics normal hairy mouse skin. This observation suggests that the hair buds achieved an attractive effect, guiding nerves to them, both *in vitro* and *in vivo*. Thus, the incorporation of hairs in tissue-engineered skin may greatly improve the recovery of the sense of touch through the combination of rapid reinnervation and an appropriate connection of sensory nerves to a sensory receptor.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Filaggrin Null Mutations Are Not a Protective Factor for Acne Vulgaris

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TO THE EDITOR

Acne vulgaris is a very common skin disorder, affecting to some degree 88–94% of Singaporean adolescents (Tan *et al.*, 2007; Yosipovitch *et al.*, 2007). Genetic predisposition is a significant risk factor, as illustrated by familial and twin studies (Bataille *et al.*, 2002; Ghodsi *et al.*, 2009). The clinical features of acne include seborrhea, comedone formation, inflammatory pustules, nodules, and cysts, with resultant scarring. Important pathogenic mechanisms in acne include increased sebum production, hyperkeratinization and occlusion of the follicular duct, proliferation of *Propionibacterium acnes*, and an inflammatory reaction (Purdy and de Berker, 2006). *P. acnes* produces lipases, which liberate proinflammatory fatty acids from sebum and also triggers a cytokine response.

Filaggrin is expressed in terminally differentiating keratinocytes and has a key role in epithelial barrier formation. Immunostaining demonstrates increased filaggrin expression in the sebaceous duct and infundibulum of acne vulgaris skin (Kurokawa *et al.*, 1988), and *P. acnes* strains increase the expression of filaggrin and other differentiation-specific markers in normal human epidermal keratinocytes *in vitro* and in the suprabasal layers of human skin explants (Jarrousse *et al.*, 2007). Similarly, inflammatory cytokines resulted in increased filaggrin expression in sebaceous gland explants (Guy and Kealey, 1998). However, it is not known whether differences in filaggrin expression represent a primary or secondary effect in the pathogenesis of acne.

Null mutations in the filaggrin gene (*FLG*) result in reduced filaggrin expression and cause ichthyosis vulgaris (Smith *et al.*, 2006). Such mutations are common in the general population, being carried by ~10% of Europeans and 7.3% of Singaporean Chinese (Sandilands *et al.*, 2007; Chen *et al.*, personal communication). This high carrier rate in different populations suggests a heterozygote advantage, and it has been proposed that a more permeable skin barrier may have been beneficial in evolutionary history (Irvine and McLean, 2006). The co-existence of *FLG* null mutations with other gene mutations that disrupt epidermal differentiation may increase phenotype severity (Liao *et al.*, 2007; Gruber *et al.*, 2009). It is also possible that heterozygosity for null mutations has other effects on skin physiology. Studying a cohort of 284 European dermatology patients not selected for dry skin (Sergeant *et al.*, 2009) raised the possibility that carriage of one *FLG* null mutation could provide a protective effect against acne vulgaris. In the Sergeant

Abbreviations: FLG, *filaggrin gene*