Betacellulin Regulates Hair Follicle Development and Hair Cycle Induction and Enhances Angiogenesis in Wounded Skin

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Betacellulin (BTC) belongs to the EGF family, whose members play important roles in skin morphogenesis, homeostasis, and repair. However, the role of BTC in skin biology is still unknown. We employed transgenic mice overexpressing BTC ubiquitously to study its role in skin physiology. Immunohistochemistry revealed increased levels of BTC especially in the hair follicles and in the epidermis of transgenic animals. Expression of key markers of epithelial differentiation was unaltered, but keratinocyte proliferation was significantly increased. At post-natal day 1 (P1), transgenic mice displayed a significant retardation of hair follicle morphogenesis. At P17, when most follicles in control mice had initiated hair follicle cycling and had already entered into their first late catagen or telogen phase, all follicles of transgenic mice were still at the mid- to late catagen phases, indicating retarded initiation of hair follicle cycling. Healing of full-thickness excisional wounds and bursting strength of incisional wounds were similar in control and transgenic mice. However, an increase in the area covered by blood vessels at the wound site was detected in transgenic animals. These results provide evidence for a role of BTC in the regulation of epidermal homeostasis, hair follicle morphogenesis and cycling, and wound angiogenesis.

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INTRODUCTION

Betacellulin (BTC) is one of seven peptide growth factors, which can directly bind and activate the EGFR (ErbB1). The other known EGFR ligands are EGF, amphiregulin (AREG), heparin-binding EGF-like growth factor (HBEGF), transforming growth factor-alpha (TGF-α), epiregulin, and epigen (Harris *et al.*, 2003). This list is most likely complete, since a genome-wide search employing algorithms based on genomic and cDNA structures failed to identify further potential EGFR ligands (Kochupurakkal *et al.*, 2005). EGFR ligands are initially synthesized as membrane-bound precursors that can be cleaved (shedded) extracellularly to release the mature, circulating form. The major effectors of this post-translational modification belong to the ADAM family of proteases (Sahin *et al.*, 2004). Interestingly, the precursor form exerts biological activity by stimulating adjacent cells via cell–cell contacts, a mechanism termed juxtacrine action (Brachmann *et al.*, 1989; Wong *et al.*, 1989; Goishi *et al.*, 1995; Higashiyama *et al.*, 1995).

The EGFR (ErbB1) is the prototype of a family of four tyrosine kinase receptors, which also includes ErbB2 (neu), ErbB3, and ErbB4. Ligand binding induces the formation of homo- or heterodimers and activation of the kinase domains (Yarden and Sliwkowski, 2001; Holbro and Hynes, 2004; Citri and Yarden, 2006). BTC, HBEGF, and epiregulin can directly activate ErbB4 in addition to ErbB1. Reflecting the essential role of ErbBs during mammalian development, complete loss of EGFR activity in knockout mice results in death either at an embryonic stage, in the perinatal period, or after a few weeks of post-natal life, depending on the genetic background (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). Knockout mice lacking ErbB2, -3, or -4 die inevitably during embryonic development (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Riethmacher *et al.*, 1997).

There is abundant evidence that normal development and homeostasis of the skin and its appendages, specifically of hair follicles, depend on the correct expression and activity of the EGFR and its ligands. EGFR, ErbB2, and ErbB3 are expressed in normal human skin (Press *et al.*, 1990; Prigent *et al.*, 1992; Hudson and McCawley, 1998; De Potter *et al.*, 2001; Stoll *et al.*, 2001; Piepkorn *et al.*, 2003) and in the HaCaT keratinocyte line (Marques *et al.*, 1999).

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Abbreviations: AREG, amphiregulin; BTC, betacellulin; HBEGF, heparin-binding EGF-like growth factor; TGF-α, transforming growth factor-alpha

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Overexpression of the EGFR has been detected in epithelial squamous cell carcinomas (Yamamoto *et al.*, 1986; Derynck *et al.*, 1987) and in psoriasis (King *et al.*, 1990; Nanney *et al.*, 1992), and substantial evidence implicates ErbB signaling as a major component in the pathogenesis of non-melanoma skin cancer (Hansen *et al.*, 2000; Sibilia *et al.*, 2000). Finally, overexpression of multiple EGFR ligands occurs in psoriatic epidermis (Elder *et al.*, 1989; Piepkorn, 1996; Piepkorn *et al.*, 1998; Stoll and Elder, 1998).

EGFR ligands are autocrine-acting growth factors for keratinocytes, playing a central role in controlling proliferation of these cells (Coffey *et al.*, 1987; Cook *et al.*, 1991; Hashimoto *et al.*, 1994; Stoll *et al.*, 1997; Piepkorn *et al.*, 1998; Shirakata *et al.*, 2000). This activity can be inhibited by blocking the EGFR kinase activity (Piepkorn *et al.*, 1994; Peus *et al.*, 1997). Interestingly, ectodomain shedding of EGFR ligands, particularly of HBEGF, was demonstrated to be essential for keratinocyte migration and efficient healing of skin wounds (Tokumaru *et al.*, 2000).

Genetically modified mouse models have been seminal in the study of the role of EGFR and its ligands in the skin and its appendages. For instance, the genetic basis of the phenotype of *waved* mice (animals displaying waved hairs and curly vibrissae known to researchers since the late 1930s) was identified as a point mutation in the *tgf-α* (wa^1) or the *egfr* (wa^2) genes (Luetteke *et al.*, 1993, 1994; Mann *et al.*, 1993). Although no obvious skin phenotype is present in $egfr^{+/-}$ mice, further decreases in EGFR activity by expression of a dominant-negative EGFR mutant (Murillas *et al.*, 1995), or the antimorphic *wa5* allele (Lee *et al.*, 2004), result in the appearance of the waved phenotype. Surviving *egfr*-knockout mice display a severe phenotype of epidermal atrophy and extremely low rates of keratinocyte proliferation (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995).

Except for TGF-α (Luetteke *et al.*, 1993; Mann *et al.*, 1993; Kim *et al.*, 2001) and HBEGF (Shirakata *et al.*, 2005), loss of other EGFR ligands failed to reveal a skin phenotype. The opposite approach, however, the overexpression of the growth factor using epithelial-specific or ubiquitous regulatory sequences, resulted in readily observable phenotypes, including wrinkled skin, alopecia and papillomas for TGF-α (Vassar and Fuchs, 1991; Dominey *et al.*, 1993), a psoriasis-like phenotype for AREG (Cook *et al.*, 1997, 2004), or arrested hair follicle development for EGF (Mak and Chan, 2003).

Although BTC is expressed in the skin, its role in skin biology is largely unknown. In human skin, BTC expression appears to be restricted to suprabasal keratinocytes, in particular to the granular cell layer (Piepkorn *et al.*, 2003; Rittie *et al.*, 2006). BTC expression is downregulated in psoriatic skin (Piepkorn *et al.*, 2003). Interestingly, treatment with retinoids markedly reduced BTC expression in human skin (Rittie *et al.*, 2006).

In this study, transgenic mice in which BTC is overexpressed ubiquitously under the control of the cytomegalovirus enhancer/chicken β-actin promoter (Schneider *et al.*, 2005) were employed to study the consequences of excess BTC in skin and its appendages. Our studies reveal a discrete, but significant perturbation of cutaneous morphogenesis and homeostasis, contrasting the more severe effects observed after overexpression of related growth factors. Interestingly, however, BTC overexpression retards murine hair follicle development and cycling, and leads to increased angiogenesis at experimental wounding sites.

RESULTS

BTC overexpression produces phenotypical abnormalities in the skin

During the initial breeding of BTC transgenic mouse line L2 (Schneider *et al.*, 2005), animals displaying wavy hairs were observed in some litters. This alteration, resembling the *waved* phenotype (Luetteke *et al.*, 1993, 1994; Mann *et al.*, 1993), was particularly evident in young animals (Figure 1a) and affected exclusively transgenic pups. The phenotype was easily visible in 10 animals (representing roughly 40% of the transgenic mice in this generation), and slight alterations were seen in another five transgenic mice. It remains unknown why further generations did not display this phenotype. Although this occasional observation may not be related to the overexpression of BTC, the known role of other EGF family members in the skin and its appendages favored this possibility. Therefore, we carefully examined the skin of additional litters at different ages. A consistent retardation in hair coat development in transgenic pups was detected. This delay was obvious between days 5 and 7 of

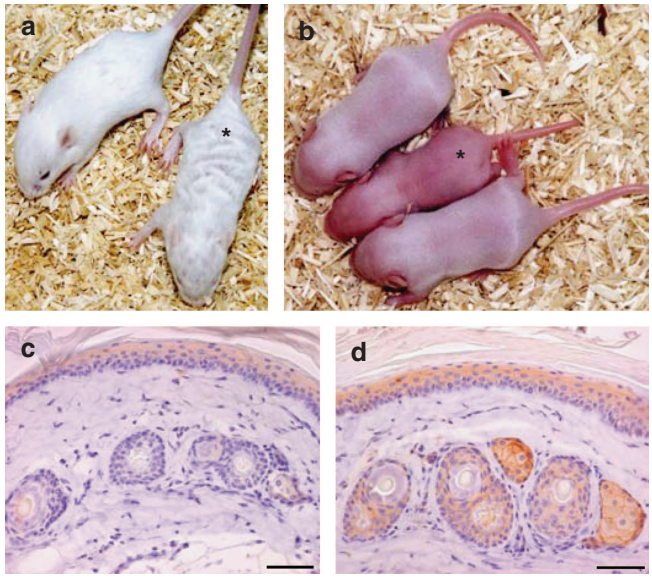

Figure 1. Skin abnormalities in BTC transgenic mice and spatial localization of the growth factor. (**a**) Waved hair was observed in some young (less than 4 weeks of age) transgenic animals (indicated by an asterisk) but not in non-transgenic littermates. (**b**) Excess of BTC caused a delay in the formation of the hair coat in transgenic pups (asterisk) as compared to control littermates. The animals shown here are 6 days old. Immunohistochemistry revealed (**c**) BTC expression in the epidermis of control mice and (**d**) increased levels of BTC in the epidermis, hair follicles, and sebaceous glands of transgenic littermates. Bar = 50 μm.

post-natal life, and was observed in every transgenic mouse of the litters examined (Figure 1b).

Endogenous and transgene-derived BTC expression in mouse skin

Although increased BTC expression in the skin of transgenic mice was detected by western blot during the initial characterization of the line (data not shown), knowledge of the site(s) of BTC overexpression within the tissue is essential for the interpretation of any phenotypical alteration. Therefore, we employed immunohistochemistry to detect BTC expression in the tail skin of transgenic mice and their control littermates. In non-transgenic mice, BTC was localized almost exclusively in the epidermis (Figure 1c). In the epidermis of transgenic mice, BTC levels were obviously increased and BTC was also detected in the hair follicles and sebaceous glands (Figure 1d).

BTC overexpression enhances keratinocyte proliferation, but does not affect their differentiation

Histological analysis of the skin of BTC transgenic mice revealed a slightly increased thickness of the epidermis. We therefore determined if this phenotype is due to enhanced proliferation and/or abnormal differentiation of keratinocytes. Localization of keratin 14 (K14) (marker for basal, proliferation-competent, non-differentiated keratinocytes), K10 (early differentiation marker), and loricrin (late terminal differentiation marker) failed to reveal any significant differences between the skin of transgenic and control animals (Figure 2a–f). Interfollicular expression of K6 is a marker for hyperproliferative and/or abnormally differentiated epidermis (Fuchs and Raghavan, 2002; Schweizer *et al.*, 2006). However, expression of this keratin was restricted to hair follicles in mice of both genotypes, and no abnormal expression was detected in interfollicular epidermis (Figure 2a–f).

Although differentiation abnormalities were not detectable, the number of BrdU-positive cells in the epidermis of transgenic mice appeared to be increased as compared to control animals (Figure 3a and b). In mice of both genotypes, proliferating keratinocytes were restricted to the basal layer. To assess this impression objectively, we measured the number of BrdU-positive cells per mm basement membrane. Our results show that keratinocyte proliferation is significantly increased in the epidermis of transgenic animals (Figure 3c). These findings demonstrate that BTC stimulates proliferation of epidermal keratinocytes but does not affect their differentiation *in vivo*.

BTC affects hair follicle morphogenesis and cycling

Next, we examined hair follicle morphogenesis during early post-natal development. At post-natal day 1 (P1), transgenic mice displayed a significantly ($P=0.01$) reduced number of follicles at stage 6 as compared to their control littermates, indicating a retardation of follicle morphogenesis (Figure 4a). At P10, this difference between transgenic and control mice had been lost and morphogenesis seemed to have catched up in the transgenic mice (data not shown). Interestingly, at P17,

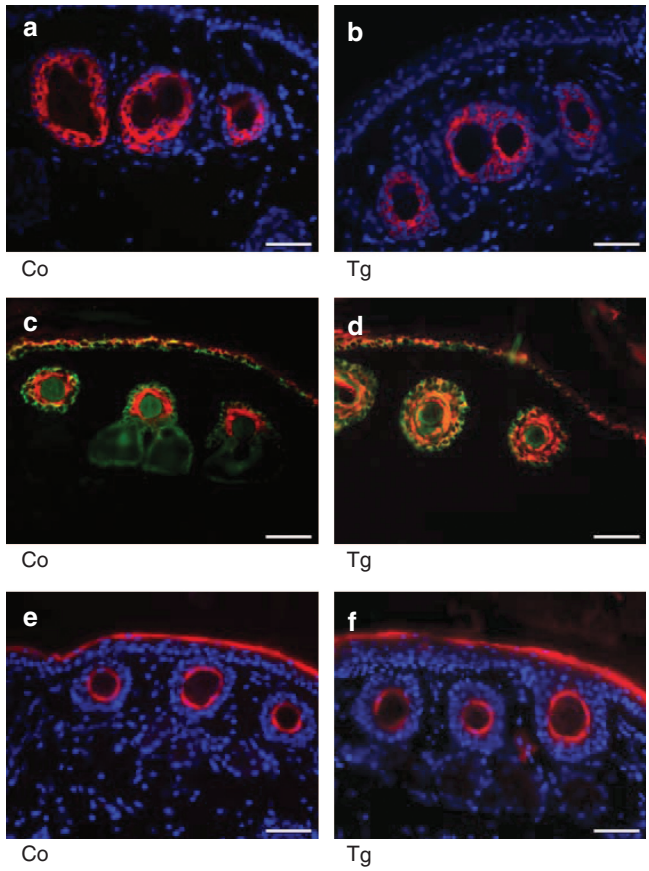

Figure 2. Keratinocyte differentiation is not affected in the epidermis of BTC transgenic mice. The expression patterns of (**a**, **b**) K6 (red), (**c**, **d**) K10 (red), K14 (green), and (**e**, **f**) loricrin (red) are similar in transgenic and control animals. Bar = 50 μm.

that is, when morphogenesis is complete and most back skin pelage follicles have entered spontaneously into hair follicle cycling by entry into their first catagen phase (apoptosis-driven hair follicle involution) (Muller-Rover *et al.*, 2001; Stenn and Paus, 2001), control hair follicles were in the late catagen phases or even already in telogen, while almost all follicles of transgenic mice were still in mid- to late catagen (Figure 4b). This finding indicates a delay in hair cycle progression (for the telogen phase $P=0.041$). Expression of BTC was also studied by immunohistochemistry at this age and revealed a similar expression pattern as in adult animals with visibly increased expression of the growth factor (data not shown).

Wound healing is not affected in BTC transgenic mice

To determine a possible role of BTC in cutaneous wound repair, we first analyzed its expression in normal skin and in full-thickness excisional skin wounds of wild-type mice by RNase protection assay. As shown in Figure 5a, BTC was expressed in normal skin and throughout the healing process, with a slight decline during the phase of granulation tissue formation (3–7 days after injury). Next, we compared expression levels of BTC in normal skin and in full-thickness excisional wounds of transgenic and control animals. In both

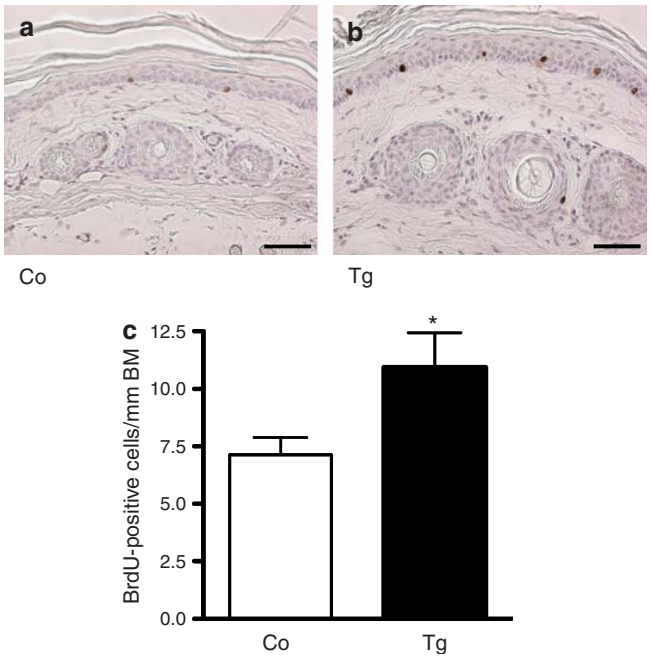

Figure 3. Enhanced keratinocyte proliferation in BTC transgenic mice. (**a**, **b**) Cell proliferation in the epidermis, measured by the number of BrdU-labeled cells (brown dots), appeared to be increased in the transgenic group. (**c**) Quantitative measurements ($n=7$, animals per group) revealed a significantly higher number of BrdU-positive cells per mm basement membrane (BM) in transgenic skin. *$P<0.05$; graph shows means ± SEM. Bar = 50 μm.

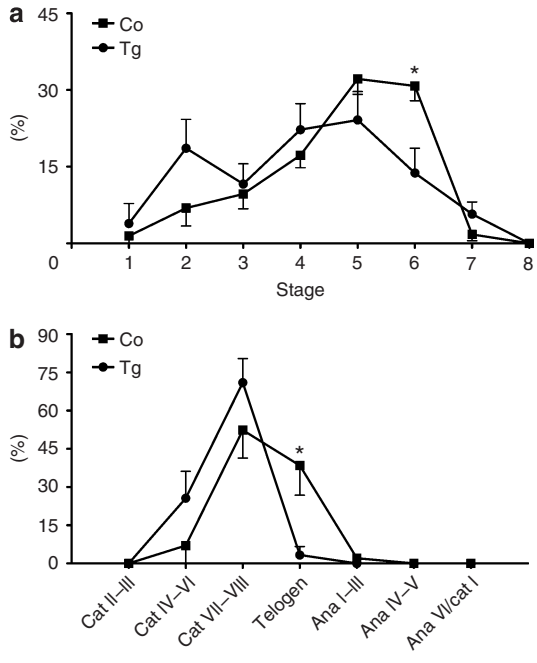

Figure 4. BTC overexpression causes a delay in hair follicle morphogenesis as well as retarded catagen progression. (**a**) At post-natal day 1 (P1), transgenic mice ($n=5$) displayed a significantly reduced number of follicles at stage 6 as compared to their control littermates ($n=5$). (**b**) At the beginning of the first hair cycle at day 17 (d17), most follicles were at the late catagen stages or telogen in non-transgenic mice ($n=8$), while nearly all follicles of transgenic mice ($n=5$) were at the mid- to late catagen phases. cat: catagen; ana: anagen; graphs show means ± SEM; *$P<0.05$.

situations, the transgene-derived BTC signal (Figure 5a, upper arrow; for explanation see Materials and Methods) was much stronger than the endogenous BTC signal (Figure 5a, lower arrow), confirming the overexpression of the growth factor in transgenic tissues (Figure 5b). Expression of BTC was predominantly observed in the hyperthickened wound epidermis as determined by immunohistochemistry (Figure 5c). In BTC transgenic mice, the staining intensity in the epidermis was further enhanced, and BTC protein was also found throughout the granulation tissue in these animals (Figure 5d). However, the enhanced levels of BTC did not obviously affect the wound-healing rate as determined by histological analysis (Figure 5d and data not shown).

Full-thickness incisional wounds were produced in a different set of animals. After 5 days, wounds were fully healed in both groups. The animals were killed at this time and the negative pressure at which the wounds rupture was measured. No significant difference in bursting strength was found in transgenic versus control mice (350 ± 40 versus 335 ± 41 mm Hg, respectively, $n=12$ wounds from 3 control and 12 wounds from 3 transgenic mice).

Increased angiogenesis at the wound site in BTC transgenic mice

Analysis of Masson trichrome-stained sections suggested the presence of a more extended vascular network in the granulation tissue of BTC transgenic mice (data not shown). To verify this impression, we first performed immunofluorescence staining of paraffin sections from day 5 wounds using an antibody against MECA-32, a marker for endothelial cells. As seen in Figure 6a–d, an increase in the number of MECA-32-positive dermal vessels was observed in transgenic mice, and the vessels appeared enlarged as compared to those in control animals. Morphometrical analysis of MECA-32-stained tissue sections (Figure 6e and f) revealed that vessel size and vessel density were indeed increased in transgenic mice, although the difference to control animals did not reach statistical significance (Figure 6g and h). The percentage of the wound area, which was covered by vessels, however, was significantly increased ($P=0.009$) in the wounds of transgenic animals (Figure 6i).

DISCUSSION

The EGFR family and its ligands play pivotal roles in the development, homeostasis, and pathology of the skin and its appendages. We have studied the effects of increased levels of the peptide growth factor BTC, a ligand of the EGFR, in the skin of transgenic mice. The main consequences of the BTC excess detected during the course of our study are (1) increased keratinocyte proliferation, (2) a discrete and reversible, but significant delay in hair follicle morphogenesis and cycling, and (3) enhanced wound healing-associated angiogenesis. These effects observed in the BTC-overexpressing mice may also give a hint to the function of the endogenous protein, since there are many examples in the literature where overexpression or inhibition of a growth factor in normal or wounded skin generated opposite phenotypes (Werner and Grose, 2003). One example is

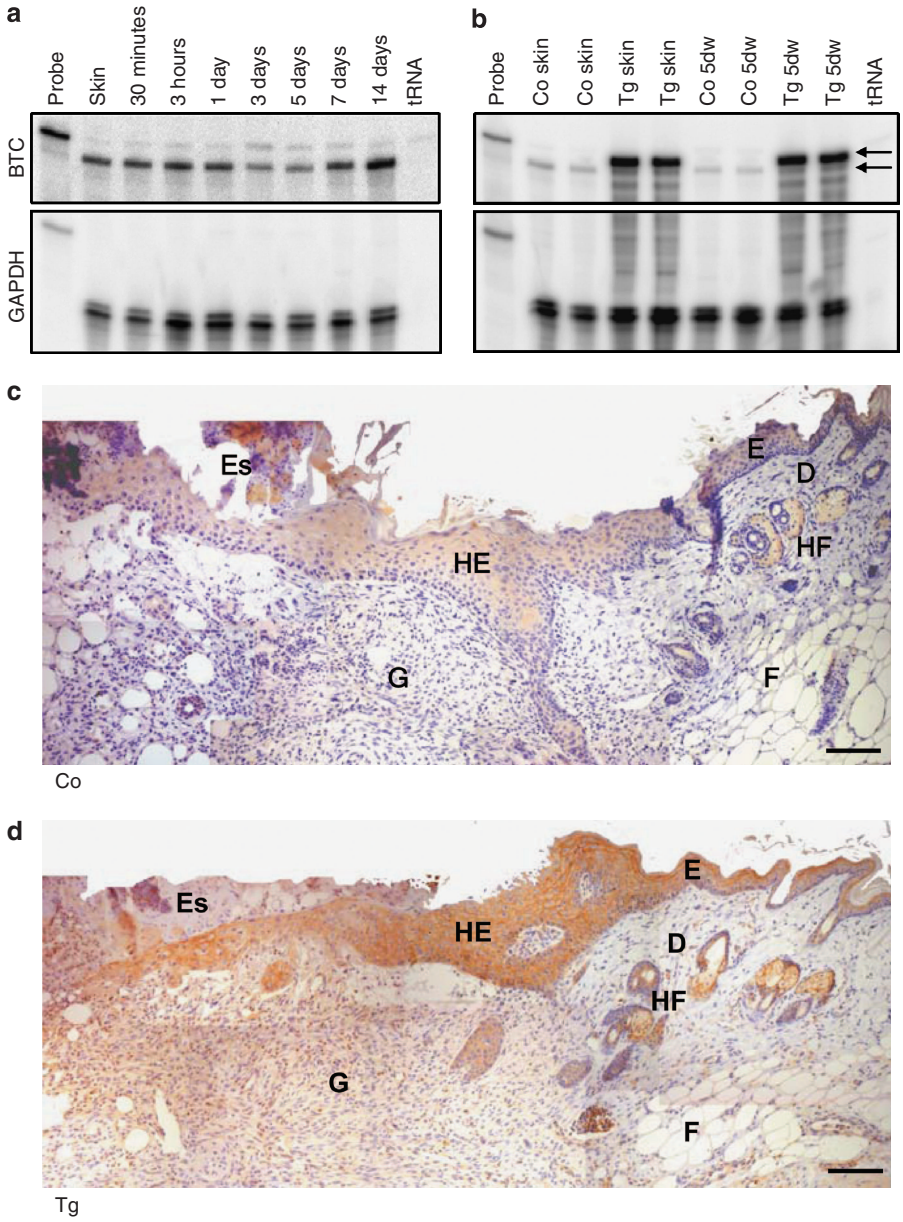

Figure 5. Expression of BTC in healing skin wounds of wild-type and BTC transgenic mice. (**a**) A 20 μg portion of total cellular RNA from normal skin (skin) and from full-thickness excisional skin wounds (30 minutes to 14 days after injury) was analyzed by RNase protection assay for the presence of BTC and GAPDH mRNAs. A 20 μg portion of tRNA was used as a negative control. As a size marker, 1,000 c.p.m. of the hybridization probes were loaded in the lane labeled ''probe.'' (**b**) RNase protection assay comparing the levels of transgene-derived BTC mRNA (upper arrow) to the endogenous BTC signal (lower arrow, slightly lower band) in normal skin and in 5-day-old wounds (5dw). Two different RNA samples from different pools of skin and wound tissue were analyzed for each time point and genotype. Immunostaining of wound sections revealed a remarkably strong expression of BTC in the hyperproliferative epithelium (HE) in both (**c**) control and (**d**) transgenic animals. (**d**) In the wound harvested from transgenic mice, BTC expression was even higher in the HE but also strong in the granulation tissue. D, dermis; E, epidermis; Es, eschar; F, fat; G, granulation tissue; HE, hyperproliferative epithelium; HF, hair follicle. Bar = 100 μm.

fibroblast growth factor 7, which caused keratinocyte hyperproliferation and epidermal hyperthickening when overexpressed in the skin (Guo *et al.*, 1993) and epidermal hypoplasia together with reduced keratinocyte proliferation when inhibited through expression of a dominant-negative receptor mutant (Werner *et al.*, 1994b). Nevertheless, functional analysis of endogenous BTC in the skin will require the analysis of knockout mice.

The increased cell proliferation observed in the epidermal compartment of BTC-overexpressing mice correlates well with the site of increased BTC expression in the skin and with the general mitogenic actions reported for BTC in a variety of cell types *in vitro* (Dunbar and Goddard, 2000). EGFR ligands are known stimulators of keratinocyte proliferation (Coffey *et al.*, 1987; Cook *et al.*, 1991; Hashimoto *et al.*, 1994; Stoll *et al.*, 1997; Piepkorn *et al.*, 1998; Shirakata *et al.*, 2000), and

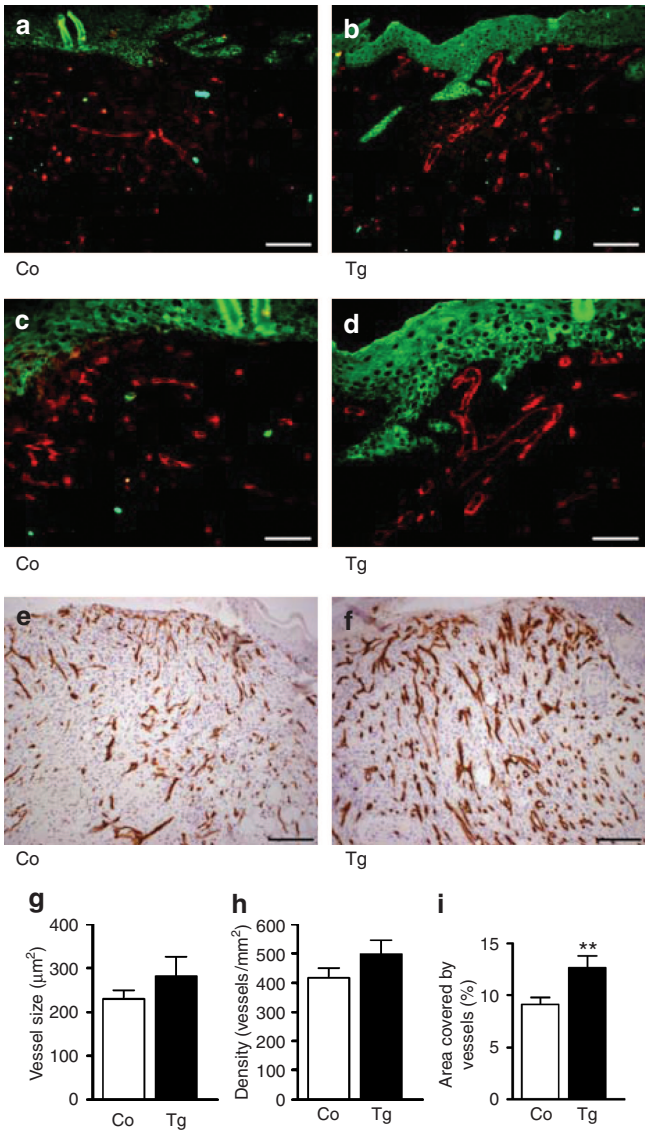

Figure 6. Angiogenesis is increased at the wound site in BTC transgenic mice. Paraffin sections of day 5 wounds evaluated by immunofluorescence staining using an antibody against MECA-32 (red) revealed a more dense vascular network in the granulation tissue of skin wounds from (**b**, **d**) transgenic mice as compared to (**a**, **c**) control animals. Counterstaining with an antibody against pan-keratin (green) was performed to visualize the wound epidermis. (**e**, **f**) MECA-32-stained sections were used for morphometrical analysis of angiogenesis ($n = 12$ wounds from 5 wild-type mice and 9 wounds from 4 transgenic mice). Vessel (**g**) size and (**h**) density values tended to be higher in wounds of transgenic mice. (**i**) The area covered by vessels was significantly increased in BTC-overexpressing mice as compared to non-transgenic littermates. $^{**}P < 0.01$; graphs show means ± SEM. Bars = (**a**, **b**, **e**, **f**) 100 μm or (**c**, **d**) 50 μm.

overexpression of AREG (Cook *et al.*, 1997, 2004) and TGF-α (Vassar and Fuchs, 1991; Dominey *et al.*, 1993) in the epidermis of transgenic mice resulted in markedly increased cell proliferation and delayed differentiation. Accordingly, reduced cell proliferation was observed in the interfollicular epidermis of *egfr*-knockout mice (Hansen *et al.*, 1996).

Along the same lines, the mild delay in hair follicle morphogenesis in BTC transgenic mice is not a surprising finding. The EGFR is essential for normal hair follicle formation and function, since reduced activity (Murillas *et al.*, 1995) or complete elimination (Hansen *et al.*, 1996, 1997) of the receptor resulted in severe architectural and functional alterations of the follicle. Altered hair follicle structure is also present in TGF-α-knockout (Luetteke *et al.*, 1993; Mann *et al.*, 1993) and in TGF-α (Vassar and Fuchs, 1991; Dominey *et al.*, 1993) and EGF transgenic mice (Mak and Chan, 2003). In fact, in a recent study, EGF was identified as the major EGFR ligand regulating hair follicle cycling. According to the model proposed by the authors, EGF may function as a switch that is turned on and off in the hair follicles, thereby regulating entry to and exit from the anagen phase (Mak and Chan, 2003). This is in line with the perturbation of hair follicle cycling observed in our transgenic mice at P17. It is conceivable that the excess of BTC favors binding of this growth factor versus other ligands to the EGFR. This may prevent the binding of the stronger keratinocyte mitogens EGF and TGF-α, thereby delaying hair follicle morphogenesis/cycling.

The EGFR ligands TGF-α, AREG, and EGF have been previously overexpressed in the skin of transgenic mice, resulting in a plethora of phenotypical alterations. While it is essential to bear in mind the limitations posed by the use of different mouse strains, different transgene expression levels, or specific cutaneous cell populations expressing the transgene, our study provides an opportunity to compare the actions of these molecules in the dermis and epidermis. EGF overexpression resulted in an arrest of hair follicle development at the final stage of morphogenesis and a transient delay in skin development (Mak and Chan, 2003). AREG overexpression caused a severe inflammatory and hyperproliferative, psoriasis-like phenotype already present at parturition (Cook *et al.*, 1997, 2004). Numerous TGF-α-overexpressing mouse lines have been generated by different laboratories during the last 15 years (Vassar and Fuchs, 1991; Dominey *et al.*, 1993; Jhappan *et al.*, 1994; Shibata *et al.*, 1997). While each model has its own specific phenotype, the bottom line can be formulated as follows: excess TGF-α in the skin causes epidermal thickening and enhances the susceptibility of the epidermis to cancer. This was particularly evident in studies where the TGF-α-overexpressing mice were treated with chemical carcinogens (Vassar *et al.*, 1992; Jhappan *et al.*, 1994; Wang *et al.*, 1994; Shibata *et al.*, 1997) or mated with other tumorigenesis-prone mouse lines like those overexpressing v-Fos (Wang *et al.*, 1999) or v-Ha-ras (Wang *et al.*, 2000) in the epidermis. The lack of hyperplasia, papillomas, or other neoplastic or metaplastic lesions in the skin of BTC transgenic mice indicates very different actions of this growth factor in the skin. This is in agreement with a previous report showing that BTC transcript levels are barely detectable in different skin tumors, indicating that this growth factor has a limited role in keratinocyte transformation (Kiguchi *et al.*, 1998).

Considering the established role of the EGFR and its ligands in wound healing and the fact that cell proliferation in

the epidermis of BTC transgenic mice was increased, it is surprising that the dynamics of wound healing and the tensile strength of healing skin wounds were not altered. It may be possible that the abundance of other EGFR ligands at the wound site (Grotendorst *et al.*, 1989) masks an effect of BTC overexpression. HBEGF appears to be the major EGFR ligand involved in wound healing: it is present at high levels in human burn wound fluid (McCarthy *et al.*, 1996) and was also identified as the principal heparin-binding growth factor in wound fluid of partial-thickness excisional wounds in pigs (Marikovsky *et al.*, 1993). HBEGF expression was detected in the advancing epithelial margin, in islands of regenerating epithelium within burn wounds, and in eccrine sweat glands (McCarthy *et al.*, 1996). It is also expressed in hair follicle epithelial cells and keratinocytes at the wound edge, particularly during the phase of keratinocyte proliferation (Cribbs *et al.*, 2002). HBEGF was also shown to be the most potent EGFR ligand in stimulating wound repair in a porcine wound model (Okwueze *et al.*, 2007). Finally, in keratinocyte-specific HBEGF-deficient mice, the migration, but not the proliferation, of keratinocytes at the wound site was impaired (Shirakata *et al.*, 2005).

The increase in angiogenesis at the wound site of BTC transgenic animals is a remarkable feature that has not been reported previously for other EGFR ligands. The psoriasis-like lesions seen in AREG transgenic mice are highly vascularized, but this is an inherent characteristic of the inflammatory process of the skin in these animals (Cook *et al.*, 1997, 2004) and may be due to overexpression of VEGF in the hyperproliferative epidermis. It has been clearly demonstrated that the EGFR is closely involved in the regulation of tumor angiogenesis (Bruns *et al.*, 2000; Karashima *et al.*, 2002). In fact, BTC itself has been implicated in angiogenesis of head and neck squamous cell carcinoma (Charoenrat *et al.*, 2000) and of hepatocellular carcinoma (Moon *et al.*, 2006). Moreover, Kim *et al.* (2003) demonstrated a strong angiogenic effect of BTC using human umbilical vein endothelial cells. These authors have identified activation of mitogen-activated protein kinase and phosphatidylinositide 3′-kinase as the major signaling pathways involved in this activity (Kim *et al.*, 2003). Thus, increased formation of blood vessels as a direct effect of BTC is feasible, in particular since BTC is highly overexpressed in the granulation tissue of our transgenic animals. However, since many other angiogenic factors, including IGF, VEGF, and TGF-β1, are abundant in skin wounds (Werner and Grose, 2003), an indirect effect cannot be excluded. Independent of its mechanisms of action, this study provided evidence for a role of BTC in wound angiogenesis.

While angiogenesis is not rate-limiting during wound healing in wild-type mice (Bloch *et al.*, 2000), it is a crucial problem in poorly healing wounds, such as in wounds of diabetic mice and patients. For example, wound angiogenesis was shown to be impaired in the genetically diabetic db/db mouse (Greenhalgh *et al.*, 1990). Topically applied VEGF reversed this phenotype through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells (Galiano *et al.*, 2004). Therefore, it will be interesting to determine if BTC has a similar effect in diabetic mice and if it can improve the healing deficiency in these animals. Together with the lack of an oncogenic effect of BTC, this may suggest the use of this growth factor for the treatment of patients suffering from poorly vascularized ulcers.

MATERIALS AND METHODS

Animals

The generation and routine genotyping of BTC transgenic mice have been described in detail previously (Schneider *et al.*, 2005). Mice used in expression studies and for phenotype analysis were weaned at an age of 3 weeks, and tail tips were used for genotyping by PCR. For the studies reported here, animals from the line L2 were used. Only hemizygous animals and their control littermates in an FVB background were employed. The animals were maintained under specific pathogen-free conditions in a closed barrier system and had free access to a standard rodent diet and water. All experimental procedures were approved by the authors' (E.W. and S.W.) institutional committee on animal care and carried out with permission from the responsible veterinary authority.

Tissue processing, immunohistochemistry, and immunofluorescence

Tail skin was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (for hematoxylin–eosin or Masson trichrome staining) or 95% ethanol/1% acetic acid (for immunohistochemistry and immunofluorescence). Immunolocalization of BTC and blood vessels was carried out with a goat polyclonal anti-BTC antibody (R&D, Wiesbaden, Germany) or with a rat monoclonal antibody against MECA-32 (BD Biosciences, San Jose, CA), respectively. Counterstaining of MECA-32-stained tissues was performed with a pan-keratin antibody (Abcam, Cambridge, UK). For immunofluorescence, ethanol/acetic acid-fixed paraffin sections (6 μm) from tail skin or wounds were incubated overnight at 4°C with the primary antibodies diluted in phosphate-buffered saline containing 3% BSA and 0.025% NP-40. After three 10 minutes washes with phosphate-buffered saline/0.1% Tween-20, sections were incubated for 1 hour with the secondary antibodies, washed again, mounted with Mowiol (Hoechst, Frankfurt, Germany) and photographed with a Zeiss Axioplan fluorescence microscope. We used rabbit polyclonal antibodies against K14, K6, loricrin (BabCO, Richmond, CA), a rat monoclonal anti-MECA-32 antibody (see above), and a murine monoclonal anti-K10 antibody (Dako, Hamburg, Germany). Secondary antibodies (coupled to Cy2 or Cy3) were from Jackson ImmunoResearch (West Grove, PA).

Detection of proliferating cells by labeling with BrdU

Mice were injected intraperitoneally with BrdU (250 mg kg^{-1}, Sigma, Buchs, Switzerland, in 1 × phosphate-buffered saline) and killed 2 hours after injection. Skin was fixed in 95% ethanol/1% acetic acid overnight and embedded in paraffin. Dewaxed 6 μm sections were rehydrated, incubated with a horseradish peroxidase-conjugated monoclonal antibody directed against BrdU (Roche, Mannheim, Germany), and stained using 3,3′-diaminobenzidine substrate (Sigma). Counterstaining was performed with hematoxylin. The sections were photographed using a Zeiss Axiophot microscope equipped with an HRC camera (Zeiss, Jena, Germany). A total of 10–13 pictures per mouse were analyzed. The number of

BrdU-positive cells was determined using the Openlab 3.1.5 software (Improvision Ltd, Basel, Switzerland) and related to the length of the basement membrane.

Hair follicle morphogenesis studies

Skin samples obtained from a defined dorsal region were dissected at the level of the subcutis, fixed in buffered paraformaldehyde (4%), and embedded in paraffin. Samples were cut parallel to the spine to obtain longitudinal hair follicle sections. Evaluation of hair follicle morphogenesis and cycle stage was performed by quantitative histomorphometry, following widely accepted guidelines (Paus *et al.*, 1999) as previously described in detail (Nakamura *et al.*, 2003; Bamberger *et al.*, 2005).

Wounding and preparation of wound tissue

Four full-thickness excisional wounds (5 mm diameter each) were made on either side of the dorsal midline of anesthetized transgenic and control females by excising skin and panniculus carnosus as described previously (Werner *et al.*, 1994a). Wounds were left uncovered and harvested 5 days after injury, bisected, fixed, and embedded in paraffin as described above. Alternatively, the complete wound including 2 mm of the wound margins was dissected and immediately frozen in liquid nitrogen for subsequent RNA isolation.

Wound bursting strength

The dorsal region of anesthetized mice was shaved and treated with a depilatory agent (Pilca Perfect; Stafford-Miller Continental, Oevel, Belgium). Two full-thickness excisions (1 cm) were made, and the skin margins were closed with strips of a wound plaster (Fixomull Stretch; Beiersdorf, Hamburg, Germany). Five days later, mice were killed and the bursting strength of the wound was determined *in situ* using the BTC-2000 system (SRLI Technologies, Nashville, TN), according to the manufacturer's instructions.

RNase protection assay

RNA isolation from wound tissue and RNase protection assay was performed as described previously (Werner *et al.*, 1993). Samples of 20 μg total RNA from normal and wounded skin at different stages after injury were used. Hybridization was performed with a ^{32}P-labeled antisense riboprobe corresponding to nucleotides 122–362 of the BTC cDNA (GI 31542237) and additional 12 nucleotides derived from the original transgene expression vector, which are therefore specific for the transgene. With this riboprobe, a 253-bp protected fragment is obtained for the transgene-derived RNA and a 241-bp protected fragment is obtained for the endogenous mRNA. Hybridization with a riboprobe corresponding to nucleotides 566–685 of the murine glyceraldehyde 3-phosphate dehydrogenase cDNA (GAPDH cDNA; NM_008084) was performed as a loading control.

Statistical analysis

Statistical significance was determined using unpaired student's *t*-tests. Differences were considered significant if $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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