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Thyroxine (T4) may promote re-epithelialisation and angiogenesis in wounded human skin *ex vivo*

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Abstract

There is a pressing need for improved preclinical model systems in which to study human skin wound healing. Here, we report the development and application of a serum-free full thickness human skin wound healing model. Not only can re-epithelialization (epidermal repair) and angiogenesis be studied in this simple and instructive model, but the model can also be used to identify clinically relevant wound-healing promoting agents, and to dissect underlying candidate mechanisms of action in the target tissue. We present preliminary *ex vivo* data to suggest that Thyroxine (T4), which reportedly promotes skin wound healing in rodents *in vivo*, may promote key features of human skin wound healing. Namely, T4 stimulates re-epithelialisation and angiogenesis, and modulates both wound healing-associated epidermal keratin expression and energy metabolism in experimentally wound human skin. Functionally, the wound healing-promoting effects of T4 are at least partially mediated via fibroblast growth factor/fibroblast growth factor receptor-mediated signalling, since they could be significantly antagonized by bFGF-neutralizing antibody. Thus, this pragmatic, easy-to-use full-thickness human skin wound healing model provides a useful preclinical research tool in the search for clinically relevant candidate wound healing-promoting agents. These *ex vivo* data encourage further pre-clinical testing of topical T4 as a cost-efficient, novel agent in the management of chronic human skin wounds.

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Abbreviations: bFGF, basic fibroblast growth factor; CK, cytokeratin; DAPI, 4'-6-diamidino-2-phenylindole; ET, epithelial tongue; FGFR1, fibroblast growth factor receptor 1; HF, hair follicle; IR, immunoreactivity; T4, thyroxine; TH, thyroid hormones; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

Introduction

Retarded healing of human skin wounds, which may result in ulceration, represents an increasing, global healthcare and quality-of-life challenge, especially in the context of an aging population [1–5]. Indeed, there is a pressing need for the identification of effective, safe and cost-efficient wound healing promoters which can be introduced into clinical practice [6]. In order to meet this need, it is critical to have simple and pragmatic, predictive model systems in which new candidate promoters of human skin wound healing can be instructively studied at the preclinical level [7, 8]. Though modelling wound healing in so-called 3D skin "equivalent" culture systems is informative [9], such systems usually lack skin appendages, immune cells, for example macrophages and mast cells, and other resident skin cells, and do not reflect the tissue tension characteristics of human skin, all of which are already known to significantly modulate cutaneous would healing [10–16]. Therefore, we and others have advocated the use of experimentally wounded full-thickness human skin *ex vivo*, ideally under defined, serum-free organ culture conditions, as well as the systematic testing of agents that have already been licensed for clinical use [7].

Thyroid hormones (THs) are of special interest in this context, since human skin and hair follicles are classical TH target organs [17–27], while thyroid diseases affect skin structure and function on multiple levels [28–30]. For example, L-thyroxine (T4) promotes human hair growth [19] and stimulates wound healing *in vivo* in rats [31] and mice [32]. Moreover, T4 operates as the chief endocrine control of amphibian metamorphosis [33], suggesting that it can act as a powerful morphogen. In addition, T4 is one of the most frequently administered hormones in clinical medicine, where it has been in extensive use for decades, its toxicology is very well-examined [34], and it is relatively inexpensive. Yet, the potential clinical utility of T4 in a dermatological setting, namely as a candidate wound healing promoter, is yet to be fully explored [30].

Given the reported wound healing-promoting properties of T4 in rodents [31, 32] and the strong interdependence of cutaneous wound healing, hair follicle (HF) cycling, HF neogenesis and skin stem cell activities [35–37], we hypothesized that T4 may also promote human skin wound healing. In order to probe whether T4 directly impacted on human skin wound healing, i.e. in the absence of other systemic/endocrine inputs, we tested our hypothesis in serum-free organ culture of full-thickness human skin [38] that had been experimentally wounded, using a "punch-in-a-punch" design [7, 39].

Recognizing that re-epithelialisation and angiogenesis are key determinants of physiological cutaneous wound healing [10, 37, 40–42] we primarily assessed T4 effects on the regenerated epidermis ('epithelial tongues' [ET]) at the inner and outer edges of wounded skin fragments [7, 43] and on intradermal angiogenesis (see Fig 1). Re-epithelialisation can be instructively quantified by planimetric measurement of both the mean length (as an indicator of keratinocyte migration) and the combined areas of the inner and outer ET (as an indicator of total epithelial regeneration) [7] (Fig 1a–1g).

Evidence for angiogenesis was assessed by quantitative CD31 immunohistomorphometry, namely CD31 immunoreactivity (IR) and the number of CD31 positive cells and cross-sectional lumina [44]. In addition, we measured the IR of basic fibroblast growth factor (bFGF, FGF2) since this pro-angiogenic growth factor is up-regulated by T4 [45, 46] and is known to play a key role as positive regulator of wound healing [47–52]. Finally, we also assessed how T4 impacts on the key wound healing associated keratin 6 (CK6) [7, 53, 54].

Material and methods

Williams' E medium (Biochrom, Cambridge, UK) supplemented with 2 mmol/liter L-glutamine (Invitrogen, Paisley, UK), 10 ng/ml hydrocortisone (Sigma-Aldrich, Taufkirchen,

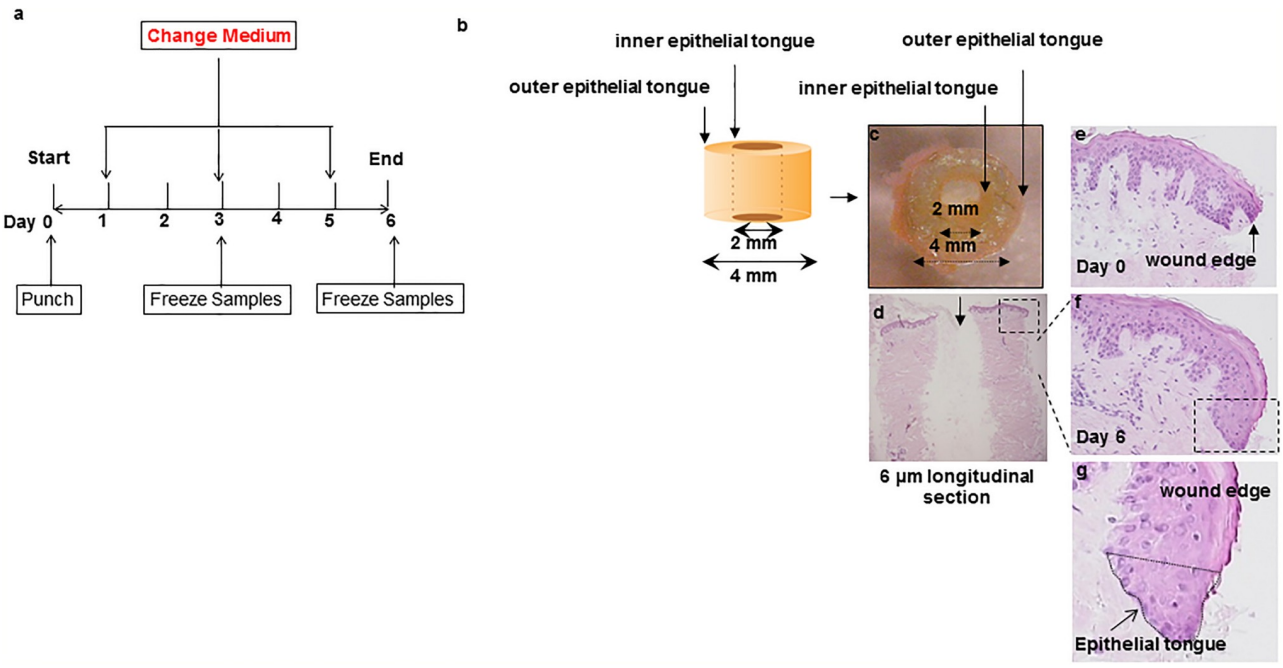

Fig 1. Establishment of human wound healing assay and testing of effects of T4 on keratinocyte migration during epidermal repair of experimentally wound human skin. (**a**) A schematic diagram of human wound healing culture model assay. A schematic diagram (**b**) and example of a wound punch sample (**c**) and longitudinal section (**d**). Sections from day 0 and day 6 of culture (vehicle control) are shown (**e**-**g**). The regenerative phenomenon is highlighted in (**f**). Magnification of marked area in (**f**) is shown in (**g**).

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Germany), 10 μg/ml insulin (Sigma), and antibiotic mixture (100 U/ml Penicillin, 10 microg/ml Streptomycin) (Sigma-Aldrich, Taufkirchen, Germany) [19, 55]. Thyroxine (T4) was obtained from Sigma. Goat polyclonal bFGF-neutralizing antibody [45] was obtained from R&D systems, Minneapolis, MN (anti-FGF basic Antibody, AB-233-NA).

Skin samples

Human scalp or corporal skin samples were obtained from patients undergoing plastic or reconstructive surgical procedures with informed consent and Institutional Research Ethics Committee permission (University of Luebeck: 06–109). The study was conducted according to The Helsinki Declaration of 1975 (revised 1983). Our study included samples from 6 patients aged from 26–67 years (average 52.6 years) (details, see Table 1).

Table 1. Characteristics of patients included in this study.

Patient	Age (years)	Sex	Location
Patient 1[a]	67	F	Temporal
Patient 2[a]	42	F	Breast
Patient 3[a]	59	F	Forearm
Patient 4[b]	61	F	Face
Patient 5[b]	61	F	Temporal
Patient 6[b]	26	M	Buttock

[a]: used for T4 experiment analysis;

[b]: used for inhibitory bFGF antibody treatment experiments

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Human skin wound healing organ culture model

The human skin wound healing assay modified based on the previously published "punch-in-a-punch" design [7, 8, 39] with the notable difference being that full-thickness (including subcutaneous fat) adult human skin was used and cultured in serum-free medium [38] (Fig 1). In the pilot study reported here, one to two "punches" were obtained for each experimental condition (control, T4 10, 100 or 1000nM) from each patient at the beginning of each culture and snap frozen at day 3 or 6 depending on the experimental group. Samples were frozen immediately for analysis (day 0) or transferred to six-well plates containing supplemented Williams E culture medium [38]. Each well contained 1–2 skin punches in 3 ml of medium. Skin samples were left untreated ("equilibration period") for the first 24 hours of the culture period. Then the medium was replaced for all samples; in the test conditions T4 was tested at concentrations ranging from 10 to 1000 nM, based on our previous human HF organ culture study [19]. Control and T4 supplemented culture medium were changed every 2 days and sample freezing was performed as per the culture protocol (Fig 1). Human skin fragments were embedded in Shandon Cryomatrix (Thermo Fisher Scientific; Waltham, MA, USA) before longitudinal cryosections (6μm) were obtained. Cryosections were stored at -80˚C until used.

Immunohistochemistry, immunofluorescence and quantitative immunohistomorphometry

The antibodies and corresponding detection methods which were used are described in Table 2. For detection of proliferating and apoptotic cells in this system, Ki67/TUNEL quantitative-immunohistomorphometry was performed as described previously [19, 23, 24, 36, 38, 56–58]. For the quantitative evaluation of the double-immunostaining results, DAPI-, Ki67-, or TUNEL-positive cells were counted in defined reference areas (see dotted line) in the newly regenerated epidermis (ETs), (Fig 1). The number of DAPI-positive cells served as "total number of cells", and the percentage of Ki-67-positive and/or TUNEL positive cells was calculated on this basis to enable comparison between control and test groups.

Standard haematoxylin and eosin staining was used to determine the new ETs. Cytokeratin 6, CD31, bFGF, and fibroblast growth factor receptor 1 (FGFR1) immunofluorescence were detected using the previously described methods [7, 19, 44, 59]. Mitochondrially encoded cytochrome c oxidase 1 (MTCO1) IR was detected by peroxidase-based avidin-biotin complex immunostaining, without counterstaining with Haematoxylin. A monoclonal antibody that

Table 2. Antibodies used for immunohistology.

Name	*Host*	*Dilution*	*Method*	*Source*	*Positive control*	*Clone*
MTCO1	Mouse	1:50	AEC	Mitosciences, Eugene, OR, USA	Skin epidermis [60, 61]	1D6E1A8
Keratin 6	Mouse	1:10	Indirect IF	PROGEN, Heidelberg, Germany	Suprabasal layers of the ORS; suprabasal layers of wounded skin [63, 79]	Ks6. KA12
bFGF	Mouse	1:50	Indirect IF	Abcam, Cambridge, UK	Epidermis [59]	ab181
FGFR1	Mouse	1:100	Indirect IF	Abcam, Cambridge, UK	Epidermis [59]	ab829
PCAM (CD31)	Mouse	1:30	Indirect IF	Dako, Glosturp, Denmark	Dermal microvessels [44]	M0823

MTCO1: cytochrome c oxidase 1; IF: immunofluorescence; TSA: Tyramide Signal Amplification; PECAM: Platelet Endothelial Cell Adhesion Molecule; CTS: connective tissue sheath

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selectively recognizes the mitochondria-specific complex IV subunit of cytochrome oxidase c was employed. [26, 27, 60, 61]. We had previously documented that the intensity of keratinocyte MTCO1 IR *in situ* correlates with the activity of respiratory chain complexes I and IV [26, 27, 60]. Given that the angiogenic effects of T4 may be mediated via upregulation of FGFR [45, 52], we cultured 1–2 "punches" of skin with either 100nM T4 or 100nM T4 plus inhibitory bFGF (8μg/ml) antibody in short-term organ culture for 3 days.

For quantitative immunohistomorphometry [23, 24, 56–58], the IR in the ETs was analysed. Both outer and inner epithelial tongue were analysed, defined as the area from the edge of the stratum corneum where the punch had been placed to the corresponding point in the epidermal basal layer. For the angiogenesis parameters, CD31 IR, the number of CD31 positive nuclei and the number of vessel lumina were determined as previously described [44]. The Image J software (National Institute of Health, Bethesda, MD) was used for evaluation. All the samples were photographed for analysis with a Keyence Biozero-8000 Microscope (Keyence Corporation, Higashi-Nakajima, Osaka, Japan).

Statistical analysis

All the data are given as mean ± standard error of the mean (SEM). For the quantitative immunohistomorphometry analyses, the IR in the ETs was measured in up to 4 sections for each wounded skin fragment. One-Way ANOVA by appropriate post hoc comparisons was used at single time points, and if the data did not follow a Gaussian distribution, non-parametric tests were applied (i.e. Kruskal-Wallis test). Statistical analysis was carried out by Graphpad prism 5.01 (Graph Pad software, Inc., San Diego, CA, USA), and $p<0.05$ was regarded as significant.

Results

After wounding, a compact sheet of epidermal keratinocytes (the ETs) began to cover the wound edges in all groups (epiboly phenomenon [62]), as expected [21]. Histochemically, overall skin morphology was well-preserved until and including day 6 of organ culture, during which time no epidermal detachment from the basal membrane was seen, while the number of proliferating or apoptotic keratinocytes in the epidermis and HFs (Ki-67/TUNEL immunofluorescence microscopy) during the entire study window of 6 days was within the expected normal range [26, 38].

Compared to the vehicle control, T4 administered to the culture medium significantly stimulated re-epithelialisation by day 3 after skin wounding in organ culture (Fig 2a–2d). Thereafter, higher concentrations of T4 rather slowed-down ET elongation (possibly due to an inhibition of keratinocyte migration). However, the total ET area (i.e. the mass of regenerated epithelium) was persistently larger in T4-treated compared to vehicle-treated wound skin fragments *ex vivo* (Fig 2a–2d).

Proliferation and apoptosis in the ETs were not significantly modulated by T4 (only high-dose T4 treatment showed a [non-significant] proliferation-stimulatory trend in the ETs at day 3), as measured by quantitative immunohistomorphometry of Ki67+ or terminal deoxynucleotidyl transferase dUTP nick end labelling-positive (TUNEL+) cells in ETs (Fig 2e–2h). This may reflect the well-recognized complexity of T4's effects on the overall tissue modelling process, which represents a balance of keratinocyte proliferation, apoptosis, differentiation and migration effects [33]. This observation suggests that the re-epithelialisation-promoting effects of T4 primarily result from the stimulation of keratinocyte migration.

T4 also significantly increased expression of the major wound healing-associated keratin, keratin 6 [53, 63] (Fig 2i and 2j), and of the mitochondrial activity protein marker, MTCO1

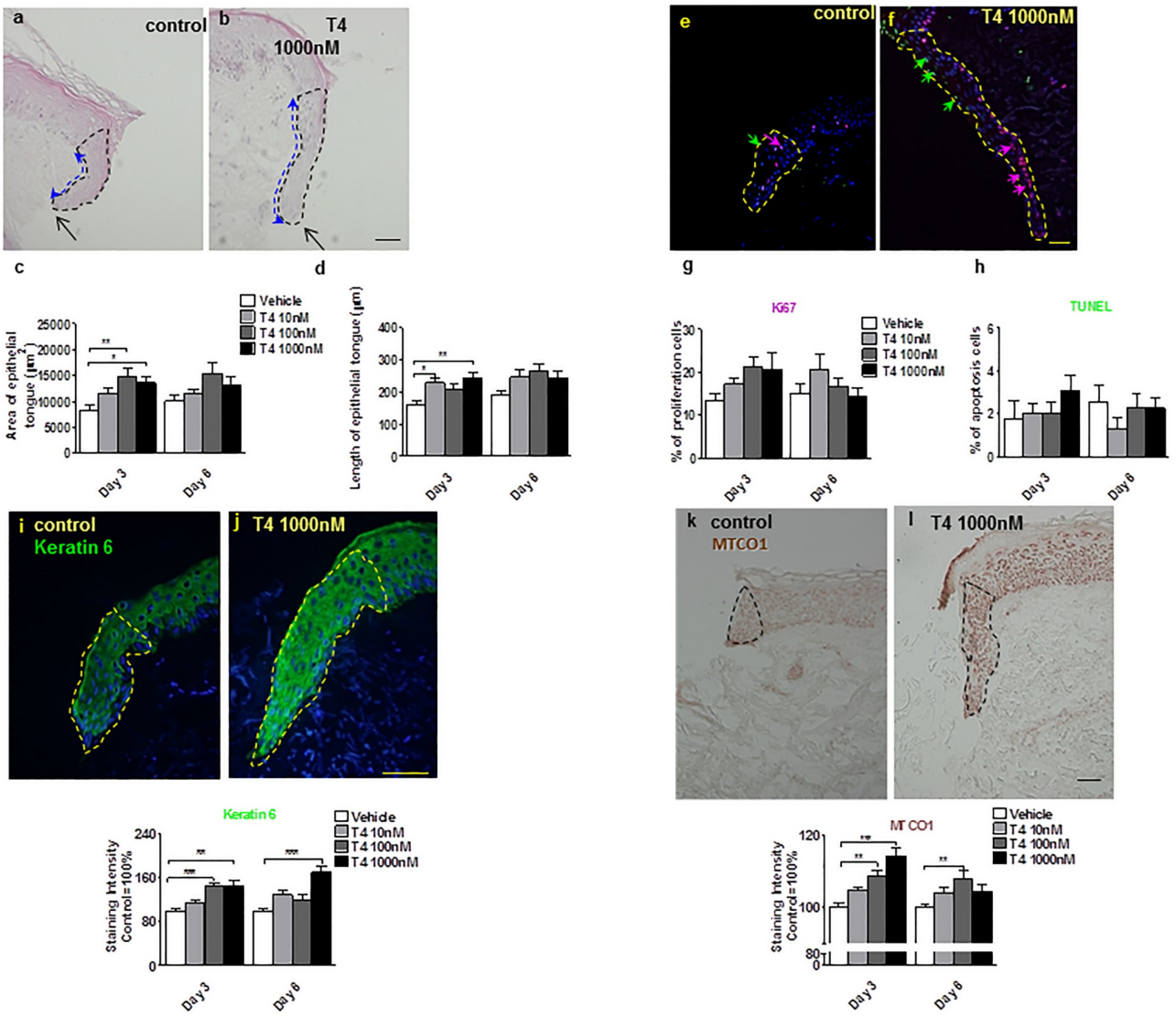

Fig 2. T4 promotes human skin re-epithelialisation. (**a-d**) Haematoxylin and eosin histochemistry: overview of wounded human skin fragment. After 3 days, epithelial tongue areas (blacked dotted line area) (ET) and length (blue dotted line) were significantly greater after treatment with T4 compared to control alone. (**e-h**) Cryosections of control-, or T4-treated human skin were examined by **Ki-67** (red arrow)/**TUNEL** (green arrow) double-labelling [19, 23, 24, 56, 57] (**e, f**). The percentage of positive cells was analyzed in the new ETs (see dotted line). When compared to the control group, more Ki-67 positive cells after 3 day culture with 1000 nM T4 treatment (and more TUNEL positive cells at day 3 in T4 1000 nM treated group were found, although the differences were not statistically significant (**e-h**). (**i, j**) **CK6** expression was significantly upregulated by T4, especially in the 1000 nM treatment group. Green fluorescence staining represented cytokeratin 6 IR in the new wound ET (dotted line area).(**k, l**) **MTCO1** expression was significantly up-regulated by T4. Brown staining represents **MTCO1** IR in the new ET. Staining intensity was measured in a defined reference area (dotted line) and normalized to the control (100%), as for CK6 expression. Number of independent experiments: n = 3 subjects (i.e. 1–2 punches per patient, per treatment group and per time point and at least 8 photomicrographs were analysed per condition); data were pooled since the results trends in all three independent experiments were comparable). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}<0.001$. Scale bars = 50μm.

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[60, 61] (Fig 2k and 2l), whose expression is well-correlated with respiratory chain complexes I and IV activity in human epidermis [26, 60, 61].

Furthermore, T4 treatment moderately increased CD31 IR, the number of CD31+ positive endothelial cells in T4-treated wounded skin fragments (Fig 3a–3e) and the microvessel density (Fig 3a, 3b and 3f), measured as described previously [44]. This suggests that T4 enhanced angiogenesis in wounded human skin fragments *ex vivo*, despite the fact that these cutaneous

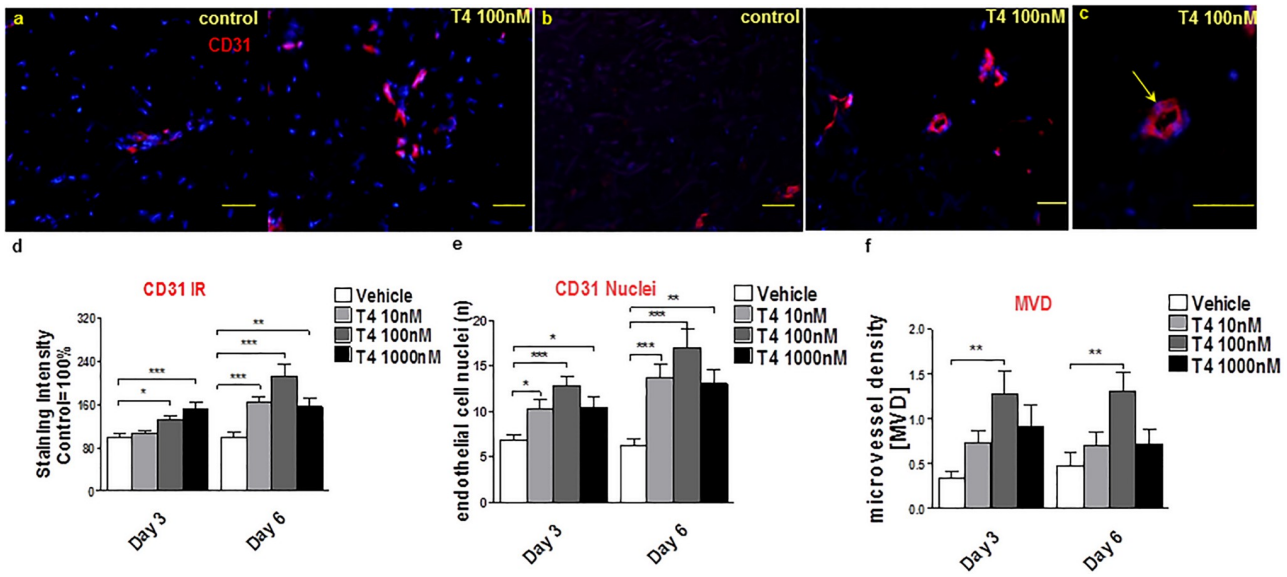

Fig 3. T4 stimulates angiogenesis in wounded human skin. (**a-c**) To analyze angiogenesis, the number of CD31+ cells (red) and of CD31+ blood vessel cross-sections (lumina) (yellow arrow, **c**) per visual field were counted by immunofluorescence microscopy (at least 12 visual fields per skin fragment were evaluated). In addition, the intensity of CD31 IR was measured. Scale bars in **a, b** = 50μm, **c** = 200μm. (**d**) CD31 IR was significantly up-regulated by T4 at days 3 and 6. Immunoreactivity data was normalized to the control data as were (**e, f**) the number of CD31 +ve endothelial cell nuclei (CD31+/DAPI+ cells) and lumina per microscopic field. Number of independent experiments: n = 3 subjects (i.e. 1–2 punches per patient, per treatment group and per time point and at least 8 photomicrographs were analyzed per condition); data were pooled since the results trends in all three independent experiments were comparable). MVD: Microvessel density; ibFGF ab: inhibitory bFGF antibody.

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blood vessels are non-perfused and may collapse to some degree after surgical skin removal. Whilst the effect was modest, it was statistically significant.

Since bFGF is a key pro-angiogenic factor known to be up-regulated by T4 [45, 46], we further examined the role of this growth factor role in T4-stimulated wounded human skin. Indeed, T4 increased bFGF protein IR in the ETs (Fig 4a–4c). Next, FGFR1 IR was assessed, since the proangiogenic actions of T4 may be indirectly mediated at least in part via up regulating FGFR1 expression [64]. In fact, as shown in Fig 4d–4f, FGFR1 was significantly increased in ETs by T4 compared to control.

Most importantly, co-administration of inhibitory-bFGF antibody [45] counteracted the stimulatory effects of T4 on re-epithelialisation (Fig 5a and 5b), CD31 protein IR (Fig 5c), and number of CD31+ endothelial cells (Fig 5d), microvessel density (Fig 5e) and bFGF expression (Fig 5f). Taken together, this suggests that the wound healing-promoting effects of T4 in organ-cultured human skin are, at least in part, bFGF-dependent.

Discussion

Here we report how organ-cultured, experimentally wounded human skin can be used to identify clinically relevant wound-healing promoting drugs, and to dissect underlying candidate mechanisms of action. The main advantage of the current model is its use of full-thickness human skin (which is readily available as excess tissue from plastic surgery) in a serum-free setting. Thus, not only the interaction between the epidermis, dermis and subcutaneous tissue is preserved, but the skin appendages, including intact HFs and sweat glands, all of which may play a substantial role in wound healing [37, 65, 66] are also present. In contrast to 3D skin culture models, it is therefore potentially possible to study the influence of the full range of resident skin cell populations on cutaneous wound healing. Future work may utilise the model to

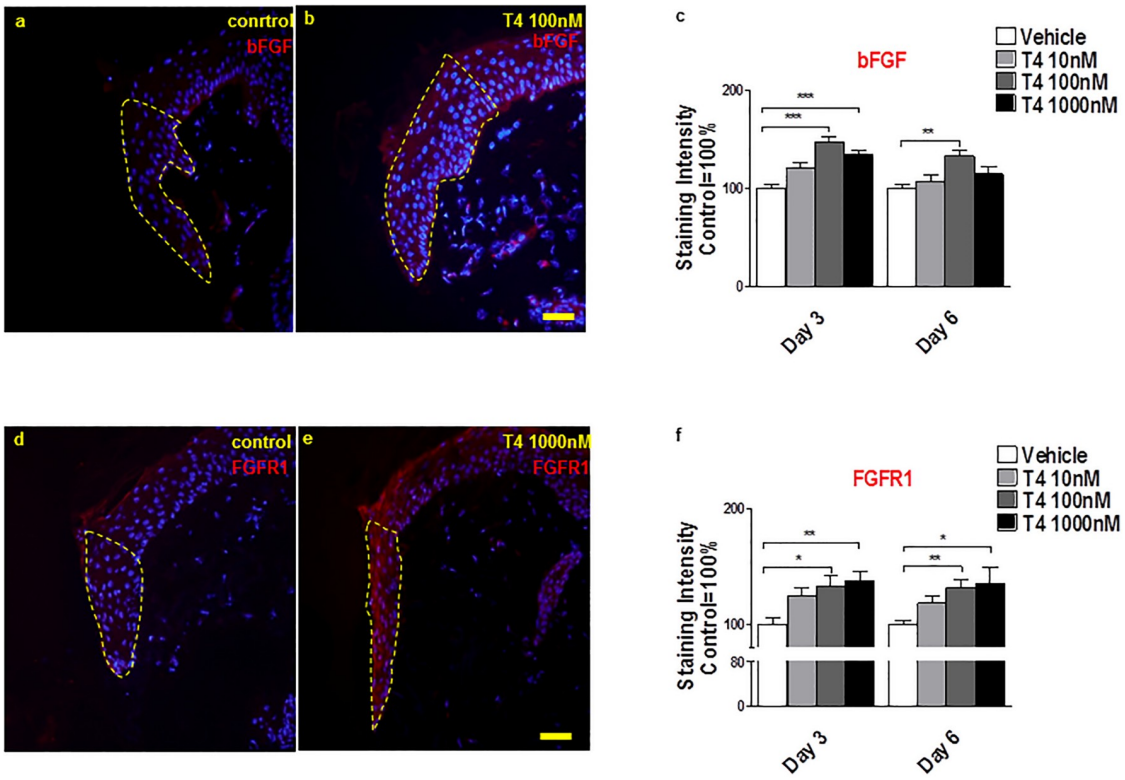

Fig 4. T4 up-regulates bFGF and FGF receptor 1 IR. (**a-c**) bFGF expression was significantly up-regulated by T4 in the epidermis of new epithelial tongues (staining intensity was measured in reference area) at day 3 and day 6 in 100nM T4 condition. Red fluorescence staining represents bFGF IR in the new epithelial tongue and the control IR value was normalized as 100%. (**d-f**) T4 increases FGFR1 expression. Red fluorescence staining represents FGFR1 IR in the new epithelial tongue. Staining intensity was measured in the dotted line reference area and the control IR value was normalized as 100%. One-Way ANOVA by appropriate post hoc comparisons was used. Data represent the mean±SEM of 3 independent experiments and normalized to the control as 100%. 1–2 punches per patient, per treatment group and per time point and at least 8 photomicrographs were analyzed per condition. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Scale bars = 50μm.

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carefully study the influence of the HF on wound healing in human skin *ex vivo*. Notably, we show *ex vivo* data to suggest that T4, a widely used, cost efficient drug with well-known toxicology, may promote key features of human skin wound healing.

The model also enables selected skin signalling pathways to be studied, just as in serum-free human HF organ culture [67–69]. Namely, provide preliminary preclinical evidence that the re-epithelialisation- and angiogenesis-promoting effects of T4 in experimentally wounded human skin *ex vivo* primarily are due to the up-regulation of bFGF/FGFR1-mediated signalling in the wounded epithelium.

Of course, it is important to also recognize the limitations of the *ex vivo* model used here. These limitations include, besides the obviously missing functional skin innervation and perfusion, the relatively short time-frame in which wound healing can be studied here. After approximately 6 days, epidermal detachment becomes a prominent feature, preventing long-term application of the model. For this reason we limited the culture time to avoid confounding the data by increasing tissue degeneration effects, and thus focused on early to medium-term interventions to promote wound healing. Another important limitation is that we used skin explants from skin containing either terminal or vellus HFs. Recently studies have confirmed the superior wound healing promoting properties of hair-bearing skin when compared

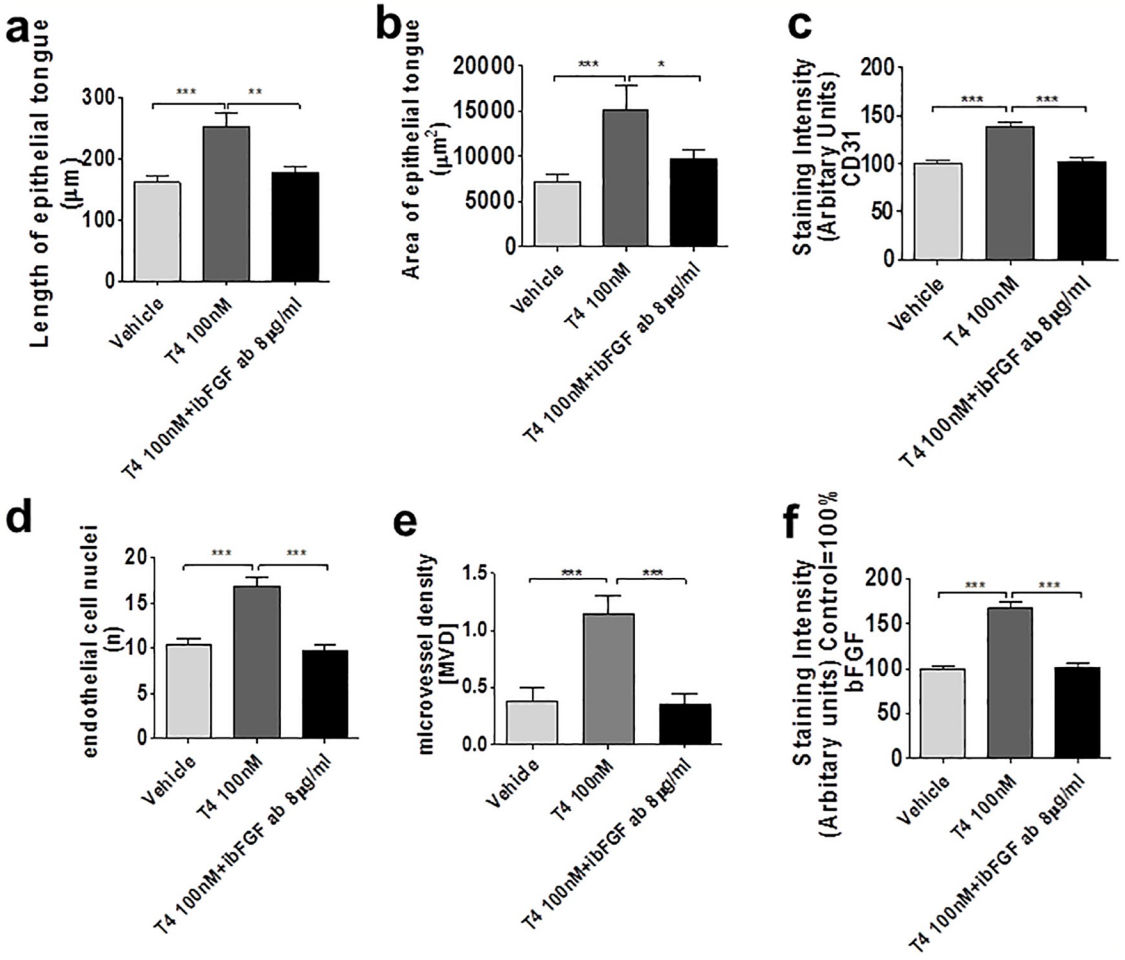

Fig 5. (a-f) T4 mediates its effect at least partially via bFGF. T4 exerts its effect partly through inducing bFGF at day 3. Area and length of the ET (**a**: length of new ET; **b**: area of new ET), intensity of the IR of CD31 (**c**), number of CD31+ cells (**d**), Number of CD31+ lumina (**e**), and intensity of IR of bFGF in new ET (**f**) were significantly increased in the T4-treated test group compared to controls. 1–2 punches per patient, per treatment group and per time point and at least 8 photomicrographs were analyzed per condition. These effects could be abrogated by co-treatment with inhibitory bFGF antibody [45].

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to skin from "hair-free" sites [65]. Whether T4 would exert superior wound healing promoting properties in terminal hair-bearing skin compared to skin dominated by vellus HFs or hair-free palmoplantar skin remains to be clarified. It must also be borne in mind that the thyroid-status of the subjects at the time of surgery was not known.

To consolidate the pilot results reported here, it is important that the current study is repeated both, with skin from an increased number of subjects and in additional wound healing models, including. for example humanized mouse *in vivo* models of wound healing [70] to confirm the positive effect of T4 on wound healing. Such pre-clinical testing is essential given the possible side-effects of topical T4 application in human subjects, included systemic absorption which might lead to a hyperthyroid state, a key consideration as increased T4 serum levels are strictly to be avoided. Reassuringly though, topical T4 may fail to influence circulating T4 levels significantly [71]. Whether the absence of perfusion-derived endocrine signalling *ex vivo* may render organ-cultured skin "functionally" hypothyroid and whether this is compensated for by increased intracutaneous conversion of residual T4 to tri-iodothyronine [19] is unknown.

Caution must be exercised with extrapolating the effects of T4 on human skin healing *ex vivo* to the more complex *in vivo* situation, as the model does not satisfactorily permit the study of the important contributions of platelets, neutrophils and circulating T cells to wound healing [72, 73]; while resident immunocytes like mast cells and macrophages are abundantly present and can be instructively studied *ex vivo* [74–77] Yet, the ex vivo effects of T4 reported here are well in line with its *in vivo* effects reported in rodent wound healing models [22, 31, 32]

Given the overall medical importance of wound healing disorders and the urgency to develop more effective, affordable, and safe wound healing-promoting agents for the treatment of chronic skin ulcers [7, 78]. Our pilot data encourage the preclinical systematic exploration of T4 as a potential wound healing promoter in experimentally wounded human skin, using additional wound healing models [8] as the next step before determining whether T4 is a plausible future skin ulcer therapy.

Supporting information

S1 Table. (Immuno)histochemical evaluations.
(XLSX)

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Formal analysis: Guo-You Zhang, Natalia T. Meier, Ralf Paus.

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Methodology: Guo-You Zhang, Natalia T. Meier, Ralf Paus.

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Visualization: Guo-You Zhang.

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Writing – review & editing: Guo-You Zhang, Ewan A. Langan, Natalia T. Meier, Wolfgang Funk, Frank Siemers, Ralf Paus.

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