Vascularised human skin equivalents as a novel in vitro model of skin fibrosis and platform for testing of antifibrotic drugs

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ABSTRACT

Objectives Fibrosis is a complex pathophysiological process involving interplay between multiple cell types. Experimental modelling of fibrosis is essential for the understanding of its pathogenesis and for testing of putative antifibrotic drugs. However, most current models employ either phylogenetically distant species or rely on human cells cultured in an artificial environment. Here we evaluated the potential of vascularised in vitro human skin equivalents as a novel model of skin fibrosis and a platform for the evaluation of antifibrotic drugs.

Methods Skin equivalents were assembled on a three-dimensional extracellular matrix by sequential seeding of endothelial cells, fibroblasts and keratinocytes. Fibrotic transformation on exposure to transforming growth factor- β (TGF β) and response to treatment with nintedanib as an established antifibrotic agent were evaluated by quantitative polymerase chain reaction (qPCR), capillary Western immunoassay, immunostaining and histology.

Results Skin equivalents perfused at a physiological pressure formed a mature, polarised epidermis, a stratified dermis and a functional vessel system. Exposure of these models to TGF β recapitulated key features of SSc skin with activation of TGF β pathways, fibroblast to myofibroblast transition, increased release of collagen and excessive deposition of extracellular matrix. Treatment with the antifibrotic agent nintedanib ameliorated this fibrotic transformation.

Conclusion Our data provide evidence that vascularised skin equivalents can replicate key features of fibrotic skin and may serve as a platform for evaluation of antifibrotic drugs in a pathophysiologically relevant human setting.

INTRODUCTION

Fibrotic disorders such as systemic sclerosis (SSc) are complex multifactorial diseases that develop as interplay of different cell types with each other and with their local microenvironment.¹ Preclinical model systems are essential to improve our understanding of the underlying pathophysiology of fibrotic diseases and to test novel therapeutic approaches. The most frequent approaches are simulating fibrotic conditions either in vitro with human cell culture systems or in vivo with mouse models. Most of the current cell culture systems

Key messages

What is already known about this subject?

- Many current model systems of skin fibrosis are of limited predictive value as they rely either on phylogenetically distant species or oversimplified culture conditions that deprive the cells of a three-dimensional matrix and of interactions with other cell types.
- These limitations provide major challenges to preclinical development and may contribute to the significant rate of negative clinical trials in systemic sclerosis (SSc).

What does this study add?

- We describe vascularised skin equivalents as an advanced model of human skin with a fully polarised epidermal layer, a stratified dermis and a functional vascular system with physiological perfusion pressures.
- Human skin equivalents can be induced to undergo fibrotic transformation and resemble key features of SSc skin with accumulation of extracellular matrix, fibroblast to myofibroblast transition and aberrant activation of transforming growth factor- β (TGF β) signalling.
- Vascularised skin equivalents can predict response to antifibrotic therapies as shown by inhibition of fibroblast activation and of extracellular matrix deposition in response to nintedanib.

How might this impact on clinical practice or future developments?

- Vascularised skin equivalents may serve as a novel model to better mimic the complex interplay of different cells in fibrotic skin and may also provide a platform for the evaluation of antifibrotic drugs in a pathophysiologically relevant human setting with improved predictive value.

are two dimensional (2D), where cells are cultured on stiff, flat plastic surfaces.² Although studies in 2D cell culture systems have provided important insights into cellular responses of individual cell types to defined stimuli, they have several

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important limitations: they cannot mimic (patho)physiologically relevant cell–matrix interactions, interactions between different cell types, presence of nutrient gradients and signalling pathways related to these processes.^{3–5} Moreover, culture of adherent cell types on plastic surfaces exposes them to an unphysiologically stiff environment, which alters central cellular responses such as response to cytokines, organisation of the cytoskeleton, migration and proliferation.^{6,7} Cellular responses in 2D culture models may thus not always reliably predict the pathological processes in fibrotic tissues. Mouse models overcome several of these limitations, such as lack of cell–cell and cell–matrix interactions, absence of gradients and excessive tissue stiffness, but contain other potential pitfalls. There are many differences between murine and human immune systems—dendritic epidermal T cells, for example, are only found in mouse.^{8,9} Moreover, not all pathways are conserved between mice and men and individual molecules may exert different functions in those signalling cascades.¹⁰ Pathophysiological studies and preclinical test programmes for novel antifibrotic approaches may thus benefit from novel in vitro test systems with primary human cells to overcome some of the limitations of conventional 2D cell culture systems and mouse models.

Here, we report an in vitro test system for skin fibrosis, generated by adapting a previously described vascularised skin equivalent.¹¹ This model employs primary human fibroblasts, keratinocytes and endothelial cells that resemble all functional skin layers affected by fibrosis in a three-dimensional (3D) environment and provides a functional vascular system with

physiological perfusion. We demonstrate that this engineered human skin equivalent can be converted into fibrotic skin by exposure to transforming growth factor- β (TGF β). The induction of fibrosis is prevented by nintedanib, demonstrating that this model is a suitable test system for candidate antifibrotic drugs. This vascularised in vitro human skin equivalent may thus be an interesting addition to the arsenal of preclinical models of skin fibrosis.

MATERIALS AND METHODS

Materials and methods are described in the online supplementary information file.

RESULTS

Vascularised human skin equivalents reproduce main characteristics of human skin relevant for the pathogenesis of skin fibrosis

We generated vascularised human skin equivalent models and evaluated their suitability as a test system for generation and prevention of skin fibrosis by exposing them to TGF β and/or nintedanib (figure 1A).¹¹

For this, we refined a previously described bioreactor design to ensure higher reproducibility and throughput. We modified the bioreactor body from a round to a rectangular shape, while adapting its inner dimensions to allow easy access to the cannulas of the artery and vein of the skin equivalent during culture (figure 1B,C,E). The polydimethylsiloxane (PDMS) lid of the

Figure 1 Assembly of the vascularised human skin equivalent and characterisation of the optimised bioreactor system. (A) Schematic overview of the generation of the human skin equivalent and its conversion to fibrotic skin. (B–D): Schematic representation of the optimised bioreactor and its contents (B, C), with the matrix at a 15° angle (D). (E) Picture illustrating the bioreactor contents: the human skin equivalent with cannulated supplying artery (red cannula) and vein (green cannula) connected to the perfusion system. (F) Picture illustrating the perfusion system and the bioreactor containing the human skin equivalent. (G) Pressure curves measured at the arterial end of the human skin equivalent (blue) following the set values (red) and resembling the physiological human systemic blood pressure. AI, arterial; BB, bioreactor body; L, lid; LF, lower frame; MR, medium reservoir; PF, pressure balance flask; PP, peristaltic pump; SE, skin equivalent; TGF β , transforming growth factor- β ; UF, upper frame; VO, venous outlet.

Figure 2 Systematic evaluation of TGF β signalling in vascularised human skin equivalent models. (A) Activation of Smad signalling as shown by immunofluorescence staining for pSMAD3 (green) and costaining with prolyl-4-hydroxylase- β (red) and DAPI (blue), at 600-fold magnification (scale bars=25 μ m). Quantification of pSMAD3 signal intensity and HE stainings (200-fold magnification, scale bars=100 μ m) is included. (B) mRNA levels of the prototypical TGF β /SMAD target genes PAI-1 and Smad7. (C) mRNA levels of ACTA2 (which encodes for α SMA). (D) Quantification of myofibroblast counts and representative α -SMA stainings counterstained with haematoxylin at 1000-fold magnification (scale bars=20 μ m). α -SMA, α -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; TGF β , transforming growth factor- β .

bioreactor provided a tight sealing while allowing direct macroscopic inspection of the skin model and its vessels during culture, which permits rapid detection of technical problems (figure 1C). Furthermore, we introduced an angled orientation of the skin equivalent to prevent entrapment of air bubbles underneath the matrix that would lead to uneven medium supply (figure 1D).

Perfusion at the arterial end was ensured by connecting the bioreactor system containing the skin models to a fluidic circuit; a peristaltic pump and a pressure sensor ensured pressure generation and control that resemble the physiological systemic blood pressure (figure 1F,G).

After 1 month of in vitro culture, the microscopic structure of the vascularised skin equivalent resembled that of human skin (online supplementary figure 1A). The keratinocytes had fully differentiated to form a polarised human epidermis with physiological location of cytokeratin-14 in the basal layer and cytokeratin-10 in the suprabasal layers and presence of a corneous layer (online supplementary figure 1B). Laminin-5 staining demonstrated that the epidermis is separated from the underlying dermis by a continuous basal membrane in our model as it is in human skin (online supplementary figure 1B). Fibroblasts migrated in the scaffold and distributed uniformly to form a dermal equivalent, while the superficial fibroblasts remain in close proximity to the epidermis.

Endothelial cells lined the pre-existent vessel structures, as shown by the positive staining for CD31 in the dermis (online supplementary figure 1C). Fibroblasts identified as vimentin-positive or P4H β -positive cells migrated in close proximity to the endothelial cells, facilitating interactions (online supplementary figure 1C). The vessel density in skin equivalent models, although lower than in human skin from

healthy donors, was similar to the density in skin from patients with SSc (online supplementary figure 2). Podoplanin-positive lymphatic endothelial cells formed lymphatic vessels in vascularised skin equivalents that persisted until the end of the experiment (online supplementary figure 3).

Exposure to TGF β induces fibroblast to myofibroblast transition and deposition of extracellular matrix (ECM) in vascularised human skin equivalents

Aberrant TGF β signalling is both sufficient and required to induce fibrosis in various organs including skin.¹² We thus evaluated whether exposure to pathophysiologically relevant concentrations of TGF β would convert normal skin equivalents to fibrotic skin equivalents.

We first aimed to confirm that incubation with TGF β activates the transcription of common downstream targets of TGF β . Indeed, we observed increased phosphorylation and nuclear translocation of Smad3 in P4H β -positive cells in the dermis of vascularised human skin equivalents (figure 2A). Moreover, the TGF- β target genes *PAI1* and *SMAD7* were upregulated in tissue homogenates from fibrotic skin equivalents as compared with the control (figure 2B).

We further analysed fibroblast to myofibroblast transition in the vascularised skin models exposed to TGF β . The mRNA levels of *ACTA2* and the number of α -smooth muscle actin (α -SMA)-positive cells were significantly higher in the TGF β -stimulated skin equivalents than in controls (figure 2C,D), thus reproducing the activated state of fibroblasts observed in fibrotic skin.¹³

We next investigated the deposition of ECM components in TGF β -exposed human skin equivalents. The mRNA levels of *COL1A1*, *COL1A2* and fibronectin were significantly increased

Figure 3 Fibrotic remodelling of the vascularised human skin equivalent in response to TGFβ. (A) Upregulation of the mRNA levels of *COL1A1*, *COL1A2* and fibronectin as major ECM proteins in the skin. (B) Representative western blot for type I collagen, with β-actin as housekeeping gene. Chemiluminescence versus molecular weight plots and quantification are included. (C) Immunofluorescence stainings of dermatan sulfate to identify human type I collagen in the vascularised human skin equivalent, at 1000-fold magnification (scale bars=20 μm), counterstained with DAPI. HE stainings (200-fold magnification, scale bars=100 μm) and quantification of collagen type I signal colocalising with dermatan sulfate are included. DAPI, 4',6-diamidino-2-phenylindole; ECM, extracellular matrix; TGFβ, transforming growth factor-β.

in the TGFβ-stimulated skin equivalents (figure 3A). Moreover, the levels of type 1 collagen protein, evaluated by capillary Western immunoassay, were highly upregulated in the TGFβ-exposed human skin equivalent (figure 3B).

However, all antibodies directed against collagen type 1 that we tested could not reliably discriminate between human (newly synthesised) and porcine (pre-existent) collagen, since collagen type 1 protein is evolutionarily highly conserved (data not shown). To identify the newly synthesised collagen type 1 and determine its relative contribution to the collagen levels in the two experimental conditions, we used an indirect approach. Dermatan sulfate was previously shown to be an excellent surrogate readout for de novo deposition of type 1 collagen, due to its association with newly formed collagen fibrils and its absence from the matrices.¹⁴ We confirmed the specificity of this approach in our setting by showing with immunofluorescence staining that collagen type 1 of porcine origin could be detected in the acellular matrix by western blot, while the dermatan sulfate signal was absent (online supplementary figure 4). The dermatan sulfate and collagen type I double positive signal highlighted the organisation of newly synthesised collagen as prominent fibres in the TGFβ group, whereas in the control group double positive signals were minimal. The intensity of collagen type I signal colocalising with dermatan sulfate was much stronger in the TGFβ group than in control, proving the increased de novo synthesis and deposition of collagen in TGFβ-exposed skin equivalents (figure 3C).

Nintedanib ameliorates fibrosis in the vascularised human skin equivalent

To evaluate its potential use as a platform to test candidate antifibrotic drugs, we analysed whether TGFβ-induced fibrosis could

be reduced by treatment with nintedanib, a multiple tyrosine kinases inhibitor with proven antifibrotic effects.^{15–17}

We first evaluated whether nintedanib can inhibit TGFβ downstream signalling and TGFβ-induced fibroblast to myofibroblast transition. In the dermis of TGFβ-exposed, nintedanib-treated skin equivalents, phosphorylation and nuclear translocation of Smad3 in P4HB-positive cells was decreased, and the TGFβ target genes *PAI1* and *SMAD7* were downregulated as compared with TGFβ-stimulated and untreated skin models (figure 4A,B). Nintedanib treatment also inhibited the TGFβ-induced upregulation of *ACTA2* mRNA levels and led to lower numbers of α-SMA-positive cells (figure 4C,D).

We subsequently analysed the deposition of ECM components in TGFβ-exposed, nintedanib-treated skin models in comparison with stimulated and vehicle-treated equivalents. TGFβ-induced upregulation of *COL1A1*, *COL1A2* and fibronectin mRNA levels and total collagen type I protein were significantly decreased by nintedanib treatment (figure 5A,B). In the TGFβ-stimulated, nintedanib-treated group, the signal intensity of newly synthesised collagen was reduced in comparison to the TGFβ-stimulated, vehicle-treated group (figure 5C).

DISCUSSION

Vascularised human skin equivalents resemble key features of human skin. The keratinocytes in the epidermal layer are polarised, and dermis and epidermis are separated by a basal membrane. Fibroblasts are located as single cells in the dermal layer, embedded into a physiological matrix. Endothelial cells form a functional, perfused vascular network comprising both blood and lymphatic vessels. This model thus includes all major resident cell types of human skin in typical topographic order

Figure 4 Inhibition of TGF β signalling by nintedanib treatment in vascularised skin equivalent models. (A) Decreased activation of TGF β -induced SMAD signalling on nintedanib treatment as shown by immunofluorescence staining for pSMAD3 (green) and costaining with prolyl-4-hydroxylase- β (red) and DAPI (blue), at 600-fold magnification (scale bars=25 μ m). Quantification of pSMAD3 signal intensity and HE stainings (200-fold magnification, scale bars=100 μ m) is included. (B) mRNA levels of the prototypical TGF β /SMAD target genes *PAI-1* and *Smad7*. (C) mRNA levels of *ACTA2* (which encodes for α -SMA). (D) Quantification of myofibroblast counts and representative α -SMA stainings counterstained with haematoxylin at 1000-fold magnification (scale bars=20 μ m). α -SMA, α -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; TGF β , transforming growth factor- β .

Figure 5 Antifibrotic effects of nintedanib treatment in vascularised skin equivalent models. (A) Inhibition of TGF β -induced upregulation of *COL1A1*, *COL1A2* and fibronectin mRNA levels by nintedanib treatment. (B) Representative western blot for type I collagen, with β -actin as housekeeping gene. Chemiluminescence versus molecular weight plots and quantification are included. (C) Immunofluorescence stainings of dermatan sulfate to identify human type I collagen in the vascularised human skin equivalent, at 1000-fold magnification (scale bars=20 μ m), counterstained with DAPI. HE stainings (200-fold magnification, scale bars=100 μ m) and quantification of collagen type I signal colocalising with dermatan sulfate are included. DAPI, 4',6-diamidino-2-phenylindole; TGF β , transforming growth factor- β .

and enables studies of interactions between fibroblasts, endothelial cells and keratinocytes. The interplay between these cell types is a critical feature of fibrotic skin diseases like SSc. Keratinocytes are a major source of proinflammatory and profibrotic cytokines in SSc, such as interleukin (IL)-1 α , IL-6, connective tissue growth factor (CTGF), oncostatin M and vascular endothelial growth factor (VEGF), and are involved in bidirectional double paracrine signalling with fibroblasts, thereby promoting fibroblast activation.^{18–21} Endothelial cells are thought to be the primary site of injury in SSc and modulate fibroblast activation by release of factors such as endothelin 1 and nitric oxide. Moreover, endothelial cells can undergo endothelial to mesenchymal transition to acquire a myofibroblast phenotype and may thus directly contribute to matrix deposition.²² Such interactions can be reproduced in vascularised skin equivalents.

We demonstrate that exposure to TGF β induces fibrotic transformation of vascularised skin equivalents. Stimulation with TGF β induces activation of classical profibrotic downstream mediators such as SMAD signalling and transcription of prototypical target genes. This promotes fibroblast to myofibroblast transition and ECM deposition in vascularised skin equivalents, key features of fibrotic skin in SSc.^{23–24} Fibroblasts in vascularised skin equivalents can therefore acquire an activated phenotype that reproduces their aberrant activation in SSc skin.

Of particular interest, we provide first evidence that vascularised skin equivalents can serve as a platform for the evaluation of antifibrotic drugs. We used nintedanib for formal proof, as nintedanib is the first targeted antifibrotic drug that awaits approval in SSc.^{15–17} Treatment with nintedanib in a pharmacologically relevant dose exerted antifibrotic effects in vascularised human skin equivalents: nintedanib attenuated TGF β signalling, reduced fibroblast to myofibroblast transition and decreased ECM deposition.

A potential alternative approach to the generation of vascularised skin equivalents from mature cell types as in our current study is the use of induced pluripotent stem cells (iPSCs). iPSCs can be rapidly expanded and can subsequently differentiated with specific protocols into the individual cell types found in human skin.^{25–30}

Although vascularised human skin equivalents may offer novel opportunities for translational studies and drug testing, the model also has important limitations: (1) The generation of vascularised skin equivalents requires specific equipment such as an incubator with adjustable perfusion pressure, bioreactors and specific material such as decellularised porcine intestinal matrices. Since adjustable perfusion pressure is an essential feature of this model, the custom incubator is indispensable; however, matrices generated by 3D bioprinters are currently evaluated as alternatives to decellularised porcine matrices and may eventually overcome the need for them.³¹ (2) Generation of vascularised skin equivalents is work and time intensive, which limits the potential for high-throughput drug screening. They thus serve as confirmatory rather than as screening models. (3) Our current version of the model aims to analyse the interaction of resident cells in the skin, but omits circulating cells such as leucocytes. However, peripheral blood mononuclear cells or selected leucocyte populations of interest can be added to the circulation of vascularised skin equivalents to study crosstalk between resident cells and circulating inflammatory cells. Experiments to study the efflux of circulating leucocytes into vascularised skin equivalents are currently in preparation.

Aside from vascularised skin equivalents, alternative in vitro approaches to mimic the complex interaction in human skin and model skin fibrosis are currently developed. Most of them

are lacking a functional vascular system.³² Given the importance of vascular cells in the pathogenesis of SSc, this may be an important limitation. Full-thickness skin models are 3D cell cultures that have an epidermal and a dermal component and reproduce the interaction between fibroblasts and keratinocytes. Such 3D models composed exclusively of fibroblasts and keratinocytes have been successfully used to study skin pathologies including fibrosis.^{33–34} Modifications of this approach that include monocytes as other pathophysiologically relevant cell types have recently been described.³⁵

In skin-on-chip models, selected skin cells are cultured in a 3D microsystem. These chips can theoretically reproduce physiological structures of different complexities up to multiorgan-on-chip.³⁶ Some of these systems include perfusion of a microfluidic channel lined by endothelial cells. However, even in these more complex skin-on-chip models, a microvascular network is lacking, thereby minimising crosstalk between endothelial cells and fibroblasts, and the physiological undulation of blood perfusion pressure cannot be reproduced.^{37–38}

Another alternative approach to in vitro generated skin models is the ex vivo culture of skin samples. In this approach, human skin samples are taken into culture directly after the biopsies have been obtained. A major advantage of this approach is that all the cellular components including immune cells and skin structures including vessels and skin appendages are preserved in their native form.^{39–41} However, the biopsies can only be kept for short period in culture, before cells lose their phenotype and undergo apoptosis. Moreover, experimental manipulation such as knockdown is challenging in this setting. In addition, standardised access to human skin samples is limited, often disabling systematic larger scale studies.

We describe herein a novel human in vitro model of skin fibrosis. Decisive features of these vascularised skin equivalents include a functional vascular perfusion system; interaction with a physiological matrix; crosstalk between the major resident cells of human skin, such as fibroblasts, keratinocytes and endothelial cells; and the option to further expand cellular interactions by the addition of leucocytes. Vascularised skin equivalents thus resemble many of the key features of fibrotic skin and may not only provide novel pathophysiological insights but also serve as an advanced platform for drug testing.

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