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RESEARCH ARTICLE

Use of versican variant V3 and versican antisense expression to engineer cultured human skin containing increased content of insoluble elastin

Mervyn J. Merrilees[1]*, Ben A. Falk[1], Ning Zuo[1], Michelle E. Dickinson[2], Barnaby C. H. May[3] and Thomas N. Wight[4]

[1]*Department of Anatomy with Radiology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, New Zealand*
[2]*Department of Chemicals and Materials Engineering, Faculty of Engineering, University of Auckland, New Zealand*
[3]*Mesynthes Ltd, 2 Kingsford Smith Drive, Auckland, New Zealand*
[4]*Benaroya Research Institute, Seattle, WA, USA*

Abstract

Skin substitutes for repair of dermal wounds are deficient in functional elastic fibres. We report that the content of insoluble elastin in the dermis of cultured human skin can be increased though the use of two approaches that enhance elastogenesis by dermal fibroblasts, forced expression of versican variant V3, which lacks glycosaminoglycan (GAG) chains, and forced expression of versican antisense to decrease levels of versican variant V1 with GAG chains. Human dermal fibroblasts transduced with V3 or anti-versican were cultured under standard conditions over a period of 4 weeks to produce dermal sheets, with growth enhanced though multiple seedings for the first 3 weeks. Human keratinocytes, cultured in supplemented media, were added to the 4-week dermal sheets and the skin layer cultured for a further week. At 5 weeks, keratinocytes were multilayered and differentiated, with desmosome junctions thoughout and keratin deposits in the upper squamous layers. The dermal layer was composed of layered fibroblasts surrounded by extracellular matrix of collagen bundles and, in control cultures, small scattered elastin deposits. Forced expression of V3 and versican antisense slowed growth, decreased versican V1 expression, increased tropoelastin expression and/or the deposition of large aggregates of insoluble elastin in the dermal layer, and increased tissue stiffness, as measured by nano-indentation. Skin sheets were also cultured on Endoform Dermal Template™, the biodegradable wound dressing made from the lamina propria of sheep foregut. Skin structure and the enhanced deposition of elastin by forced expression of V3 and anti-versican were preserved on this supportive substrate. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords **cultured skin; human; epidermis; dermis; elastin; versican V3**

1. Introduction

Various approaches are currently available for restoring cover to skin wounds, including the use of autologous cultured keratinocytes and dermal fibroblasts, the latter frequently seeded into or onto dermal substitutes consisting of acellular collagen scaffolds (Böttcher-Haberzeth *et al*., 2010; Boyce and Warden, 2002; Pham *et al*., 2007). Despite recent improvements in outcomes for epidermal–dermal combinations, none of these approaches has been able to provide a covering that matches the structural and functional properties of normal skin. Recreating the dermis has proved particularly difficult and often the long-term result is formation of scar tissue that has a high content of collagen and a deficiency of elastic fibres (Rnjak *et al*., 2011). Elastic fibres, in combination with

*Correspondence to: Mervyn J. Merrilees, Department of Anatomy with Radiology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand. E-mail: m.mmerrilees@auckland.ac.nz

collagen, have long been recognized to play a key role in providing balanced mechanical properties by imparting restrained expansion under load (Burton, 1954) and diminished restraint, with increased tensional load on the collagen, favours scar formation (Flint, 1976). The question arises as to whether or not it is possible to culture a skin graft with an increased content of elastin, and within a clinically acceptable time frame.

Dermal fibroblasts in skin that is deficient in elastin do not have an impaired capacity for synthesis and secretion of tropoelastin (Hinek *et al*., 2000); rather, it is the assembly and crosslinking of tropoelastin on the microfibrillar scaffold of elastic fibres that does not take place. Assembly is facilitated by the chaperone elastin binding protein (EBP) that delivers tropoelastin to the cell surface, but in the presence of pericellular proteoglycans, such as versican, with constituent chondroitin sulphate (CS)-rich glycosaminoglycan (GAG) chains, the transfer of tropoelastin from EBP to the microfibrils, and the subsequent lysyl oxidase-mediated crosslinking, is inhibited (Hinek and Wilson, 2000; Hinek *et al*., 2000).

In previous studies we demonstrated, *in vitro* and *in vivo*, that overexpression of versican variant V3 that lacks CS chains, or overexpression of an antisense sequence against versican variants with chains (V0, V1, V2), leads to enhanced deposition of mature crosslinked elastic fibres (Huang *et al*., 2006; Merrilees *et al*., 2002). Similarly, removal of CS chains by chondroitinase ABC treatment can reverse impaired elastogenesis (Hinek *et al.*, 2000). *In vivo*, overexpression of V3 (Merrilees *et al*., 2002, 2011) and versican antisense (Huang *et al*., 2006) by transduced smooth muscle cells seeded onto ballooned intima of vessels results in organized layers of mature elastic fibres thoughout the newly formed neointima. Overexpression of both V3 and versican antisense result in a significant decrease in the accumulation of CS in the pericellular matrix and a corresponding increase in EBP, indicating that the composition of the matrix in the pericellular zone, where fibre assembly takes place, is a significant determinant in achieving the final phase of elastogenesis and the deposition of insoluble elastin (Huang *et al*., 2006; Merrilees *et al*., 2002, 2011).

In this study we transduced human dermal fibroblasts with the gene for V3 and a gene encoding antisense against versican, to determine whether it is possible to increase the content of insoluble elastin in cultured skin grafts. We demonstrate that cultured skin sheets, produced over 5 weeks and composed of both a dermis produced by fibroblasts expressing V3 and an epidermis of keratinocytes, do contain an increased content of insoluble elastin, and show that the sheets have altered mechanical properties. We further show that the skin sheets can be cultured on the biodegradable wound dressing Endoform Dermal Template™, made from the lamina propria of sheep foregut, and that expression of V3 and anti-versican by fibroblasts enhances deposition of elastin in dermis formed on this supportive substrate.

2. Materials and methods

2.1. Cells and retroviral transduction

Human neonatal dermal fibroblasts (hnDFs) and human neonatal primary epidermal keratinocytes (hnKs) from ATCC® Primary Cell Solutions™ were cultured according to protocols provided (see ATCC® PCS-201-010, ATCC® PCS-200-010). Full-length sense and antisense V3 sequences were used to construct retroviral vectors, using methodology described previously for V3 (Clowes *et al*., 1994; Lemire *et al*., 2002). Briefly, rat V3 cDNA and the complementary product were each inserted into the *Bam*HI site of empty retroviral vector LXSN to produce V3 and versican antisense-containing vectors, respectively. The retroviral vector containing the V3 gene and the versican antisense sequence, as well as the empty control vector, were used to transduce cultured hnDFs using PA317 packaging cells, as previously described. Transduced cells were selected using the neomycin analogue G418. Expression levels of V3 using this system have been documented previously for vascular smooth muscle cells *in vitro* (Lemire *et al*., 2002) and *in vivo* (Merrilees *et al*., 2002) and for human skin fibroblasts (Hinek *et al*., 2004). In these cells, V3 expression levels were similar to or greater than expression of V1 transcripts. Cells between passages 3 and 7 were used for experiments.

2.2. Cell-seeding and multiple-layering protocol

Vector control, V3 and anti-versican transduced hnDF were seeded into six-well plates (BD Falcon™ cat. no. 353502) at a density of 5×10^5 cells/well in 5 ml Dulbecco's modified Eagle's medium (DMEM)-high glucose (Invitrogen cat. no. 10569–044), supplemented with 10% fetal bovine serum (FBS; Thermo HyClone cat. no. SH30406.02) and glutamine pen–strep (Invitrogen cat. no.10378-016). Further seedings of the respective hnDF lines were performed twice weekly for 3 weeks. Following a further week of culture without additional hnDF seedings, hnKs, cultured in EpiLife Medium (Invitrogen cat. no. M-EPI-500-CA) with human keratinocyte growth supplement (HKGS; Invitrogen cat. no. S-001-5), were seeded onto the multilayers of vector control, V3 and anti-versican hnDF, at a density of 5×10^5 cells/well and maintained in 5 ml/well DMEM-high glucose medium supplemented with HKGS (1%). Thoughout, the culture medium was changed at each seeding (weeks 1–3) and in the final 2 weeks every 3 days.

2.3. Cell proliferation

Vector control, V3 and anti-versican hnDF were seeded into 12-well plates (BD Falcon™ cat. no. 353502) at a density of 2.5×10^5 cells/plate, supplemented with 10% FBS and glutamine pen–strep. Cell numbers were determined using a haemocytometer on days 0, 1, 2, 4

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and 7. Growth rate was determined as percentage increase of count on day 0. Counts were in triplicate for each time point.

2.4. Endoform carrier

Endoform Dermal Template™ sheets (4 × 4 cm) from Mesynthes, New Zealand, were mounted between two frames (4 × 4 cm, with margins 5 mm wide), secured by four screws at each corner of the frames. Prior to culture, the two frames were sterilized in 70% ethanol and dried for 2 h in a biohazard safety cabinet. The Endoform sheets, provided in a sterile pack, were further sterilized by UV for 15 min on each side in a biohazard safety cabinet before mounting between the frames. Frames with mounted Endoform were soaked in DMEM-high glucose for 24 h prior to cell seeding. The medium was replaced and the cells seeded carefully onto the Endoform sheets. Cell attachment on the Endoform sheets was facilitated by leaving the seeded Endoform sheets in a biohazard safety cabinet for 10 min before transferring to a 37°C CO_2 incubator for further culture.

2.5. Histochemistry and immunohistochemistry

2.5.1. Elastin and chondroitin sulphate

Vector control, V3 and anti-versican hnDF, seeded at a density of 5×10^5 cells/well into six-well plates, followed by multiple similar seedings twice weekly up to 4 weeks, were fixed in 4% paraformaldehyde at weeks 2 and 4 (huDFs alone) and at week 5 (hnDFs + hnKs). Week 4 dermal sheets were immunostained *in situ* and week 5 skin sheets detached, processed and embedded in paraffin, and histological and immunostaining performed on 10 μm sections. Deparaffinized sections were stained with haematoxylin and eosin (H&E) and elastin van Gieson to display general tissue morphology and elastin organization, respectively. For immunohistochemistry, deparaffinized sections were hydrated in phosphate-buffered saline (PBS) for 3 × 5 min, blocked with 1% donkey serum for 1 h and then incubated with primary polyclonal rabbit elastin antibody (1:500; Calbiochem® cat. no. 324756) and anti-chondroitin sulphate supernatant [1:10; Developmental Studies Hybridoma Bank (DSHB), cat. no. 9BA12] at 4°C overnight. The sections were then washed for 2 × 5 min in PBS, incubated for 1 h in secondary antibodies Alexa 594 goat-anti-rabbit IgG at 1:500 (Invitrogen cat. no. A-11012) and Alexa 594 goat-anti-mouse IgG at 1:500 (Invitrogen cat. no. A-11005), respectively. The slides were rinsed in PBS and mounted with ProLong® Gold Antifade Reagent with DAPI (Molecular Probes® cat. no. P36935). Immunohistochemistry was also carried out on the fibroblast multilayers *in situ* in the six-well plates at 2 and 4 weeks.

2.5.2. Keratin

Vector control, V3 and anti-versican hnDF were seeded at a density of 5×10^5 cells into six-well plates and into framed Endoform, twice a week for 4 weeks, fixed in 4% formaldehyde, processed and sectioned, washed in PBS, blocked in 1% donkey serum and incubated for 2 h with primary keratin antibody VM-1 (1:250; DSHB), washed in PBS and incubated for 1 h in secondary antibody Alexa 594 goat-anti-mouse IgG (1:500; Alexa cat. no. 594). The slides were rinsed in PBS and mounted with ProLong® Gold Antifade Reagent with DAPI.

2.6. Morphometry

Fluorescent intensities (pixel areas) of week 4 dermal sheets, stained for elastin and CS, were determined using ImageJ and split-channel and thesholding analysis. Volume fractions for elastin in skin sheets cultured on Endoform Dermal Template™ were determined by point counting (100 point grid), as previously described (Black *et al.*, 2008), of images (×100 objective) of immunofluorescently stained elastin.

2.7. Electron microscopy

Skin sheets for electron microscopy were fixed in 4% paraformaldehyde, post-fixed in 1% OsO_4, processed and sectioned transversely. Thin sections, stained with uranyl acetate and lead citrate, were viewed and photographed on a Tecnai™ G^2 Spirit Twin transmission electron microscope.

2.8. qRT–PCR tropoelastin

Total RNA from cultures of vector control hnDF, V3 and anti-versican hnDF, at weeks 2 and 4, from the 5-week cultures of huDFs + hnKs, and from keratinocytes cultured alone for 7 days, was extracted using PureLink® RNA Mini Kit (Invitrogen cat. no. 12183018A). Total RNA (1 μg) from each sample was transcribed using a SuperScript® ViLO cDNA synthesis Kit (Invitrogen cat. no. 11754–050) according to the manufacturer's instructions. Real-time PCR was performed using Express SYBR® GreenER™ qPCR Supermix with Premixed ROX (Invitrogen cat. no. 11794–200), in accordance with the manufacturer's instructions. ABI 7900HT Fast Real-Time PCR System (Applied Biosystems® cat. no. 4329002) was used, with cycler conditions, as follows: incubation for 2 min at 50°C, followed by another incubation step at 95°C for 2 min, then 1 min at and 60°C and 15 s at 95°C for 40 cycles. The relative mRNA expression of target genes in each sample was quantified and normalized to the *GAPDH* mRNA levels, using the $2^{-\Delta\Delta C_T}$ method. The primers used were: 5′-TGGAGTTCCAGGTGTTGGGGGC-3′ as the elastin forward primer; and 5′-AAGCCAGGTCTTGCTGCCACTCCG-3′ for the reverse primer.

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2.9. Fastin™ assay for insoluble elastin

Vector control, V3 and anti-versican hnDFs (5×10^5/well) were seeded in six-well plates, with further seedings twice weekly for 3 weeks. Following a further week of culture without additional seedings, the elastin content of the cells was quantified using a Fastin™ Elastin Assay Kit (Biocolor Ltd, UK). Briefly, insoluble elastin was solubilized by hot oxalic acid treatment, precipitated and mixed with the Fastin dye reagent. The elastin–dye complex was collected by centrifugation and dye bound to the pellet solubilized with the dissociation reagent and read in a six-well plate, using a microplate reader set to 513 nm. All measurements were performed in duplicate. Measured elastin amounts were normalized to the corresponding wet weights of cell sheets.

2.10. Mechanical testing

Mechanical properties of skin sheets were determined by testing on a Hysitron TI-950 TriboIndenter. Small sheets (1.5×1.5 cm) were secured between two plastic plates, each with an open round window (~5 mm diameter) and the lower with a raised rim around the opening that fitted within the hole of the upper plate. Skin sheets were placed over the raised rim, the upper plate placed over the rim, such that the sheet was taut but not stretched, and the two plates secured together with tapping screws. The fixture was immersed in PBS and mounted in the TriboIndenter for testing. Multiple testing runs for displacement were conducted for vector control, V3 and anti-versican skin sheets, using a 20 μm diameter probe and forces in the range 1–7 μN.

2.11. Statistical analyses

Data were analysed by Student's *t*-test. A value of $p < 0.05$ was taken as significant.

3. Results

3.1. Culture of skin sheets

Vector control, V3 and anti-versican transduced dermal fibroblasts showed morphological differences between cells that were most apparent 48 h after the initial seedings. Compared with the typical elongated spindle-shaped vector control cells, V3 cells were more flattened and spread, features more evident in anti-versian cells (Figure 1a–c). These changes were reflected in growth, with anti-versican transduced cells having the slowest growth rate (Figure 1d). These differences in morphology and growth were similar to those observed for vascular smooth muscle cells similarly transduced (Lemire *et al.*, 2002). Following multiple cell seedings for 3 weeks and growth for a further week, fibroblasts formed multilayered tissue sheets, with cells in each layer aligned in parallel orientation but varying in direction between layers, to create mostly orthogonal arrays (Figure 1e). Keratinocytes seeded on top of the fibroblast sheets were initially scattered, but over the 1-week culture period frequently formed distinct islands of cells (Figure 1f). As described below, multilayers of keratinocytes were present 1 week after seeding. At 5 weeks the protocol of multiple seedings resulted in bilayer sheets of skin with a dermis and epidermis, although with no apparent visual differences between control, V3 or anti-versican sheets (Figure 1g, h), although the anti-versican transduced fibroblast sheets were generally thinner and more adherent to the culture substrate.

3.2. Structure of skin sheets

Histological sections of skin sheets showed distinct keratinocyte and a fibroblast layers with a fibrous dermis (Figure 2a, b). Keratinocytes at 1 week showed evidence of differentiation and were immunopositive for keratin (VM-1 staining; Figure 2c, d), both in the control (Figure 2c) and V3 skin sheets cultured on plastic (V3 data not shown, as identical to controls) and on Endoform Dermal Template™ (Figure 2d). Differentiation was confirmed by electron microscopy, with keratin granules present in the upper layers of flattened keratinocytes in control and V3 cultures and desmosome junctions present thoughout the forming epidermis (Figure 2e). The dermis was characterized by scattered and mostly elongated fibroblasts (subjectively with more elongation in V3 dermis) in an extracellular matrix containing mostly small-diameter collagen fibres. At the dermal–epidermal junction, basement membrane formation was evident (Figure 2f).

Immunofluorescent staining for elastin revealed a significant difference between vector control and V3 dermis. Vector control sheets showed elastin present as small isolated deposits (Figure 3a). V3 sheets, and to a lesser extent anti-versican sheets (data not shown), showed large aggregations of elastin (Figure 3b), interpreted as the beginnings of fibre formation. The increase in elastin content in the V3 skin sheets was confirmed by extraction of elastin from the cell sheets and determination of insoluble elastin content by the Fastin™ assay (Figure 3c). Antisense skin sheets showed a small but not significant increase. Electron microscopy showed that whereas collagen dominated in the extracellular matrix of dermis formed by vector control cells (Figure 3d), V3 dermis contained prominent deposits of elastin, notably close to the under-surface of fibroblasts, a feature noted previously for elastin deposition by cells in culture (Merrilees *et al.*, 2002).

3.3. Reciprocal relationship between elastin and chondroitin sulphate

Previous studies have reported that elastin (insoluble) and chondroitin sulphate show a reciprocal relationship

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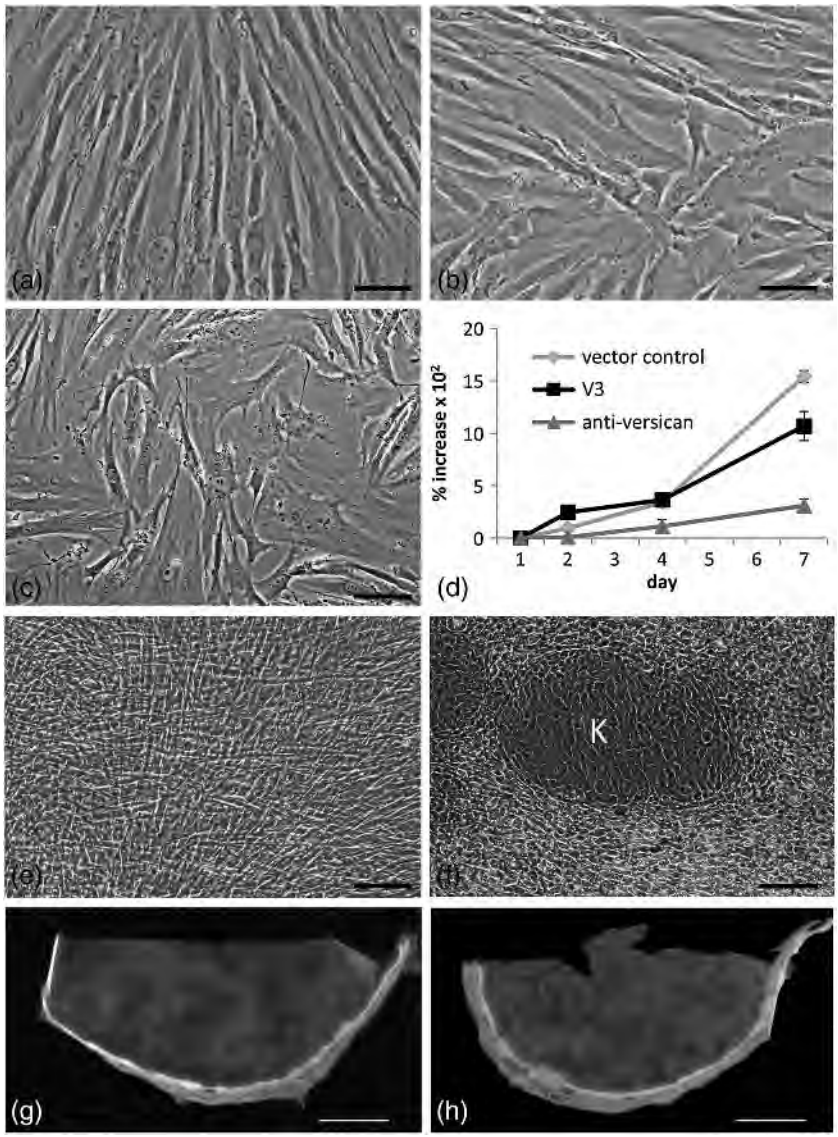

Figure 1. (a–c) Cultured human dermal fibroblasts at low density, 48 h after initial seeding, showing flattened morphology of cells transduced with V3 (b) and anti-versican (c) compared with empty vector (a). (d) Comparative growth rates of vector control, V3 and anti-versican-transduced fibroblasts over 1 week of growth from a single seeding. (e) Multilayered vector control fibroblast sheet at 4 weeks. (f) Multilayered V3 fibroblast sheet at 5 weeks, showing an island of keratinocytes (K) seeded at week 4; individual keratincytes (round dark cells) are also scattered over underlying fibroblasts. (g, h) Vector control (g) and V3 (h) skin sheets at 5 weeks (fibroblasts plus keratinocytes), detached from six-well culture plates and cut in half; light zone of semicircular peripheral tissue is folded skin detached from well margins. Magnification bars = (a–c) 25 μm, (e, f) 100 μm, (g, h) 1 cm

(Hinek *et al.*, 2000; Huang *et al.*, 2006; Merrilees *et al.*, 2002), consistent with chondroitin sulphate inhibition of elastin assembly though interference with EBP-mediated delivery of tropoelastin to nacent elastic fibres at the cell surface (Hinek and Wilson, 2000; Hinek *et al.*, 2000). To determine whether a similar inverse relationship was present for the dermal skin sheets, 4-week fibroblast sheets were immunostained for elastin and chondroitin sulphate. A reciprocal relationship was observed, with low levels of elastin immunoreactivity associated with strong CS staining in the vector control sheets, and correspondingly increased elastin staining in V3 and anti-versican associated with reduced staining for CS (Figure 4). ImageJ analysis of pixel areas for immunofluorescent elastin and CS confirmed the subjective evaluation of the reciprocal relationship (Figure 4).

3.4. Tropoelastin and versican V1 mRNA levels

To determine whether the changes in insoluble elastin and versican were reflected in changes in mRNA expression, qRT–PCR was used to determine mRNA levels for fibroblasts alone (weeks 2 and 4 for elastin, week 4 for versican), fibroblasts plus keratinocytes (week 5) and keratinocytes alone (7 days). Tropoelastin mRNA levels in V3 cultures at 2 and 4 weeks showed small but significant decreases compared to vector control cultures, but not at week 5; anti-versican cultures showed increased message levels at weeks 2 and 4, but not at week 5 (Figure 5). Message levels in the fibroblasts trended upwards, for all groups, thoughout the culture period. Keratinocytes cultured alone showed low but detectable levels of elastin message (Figure 5).

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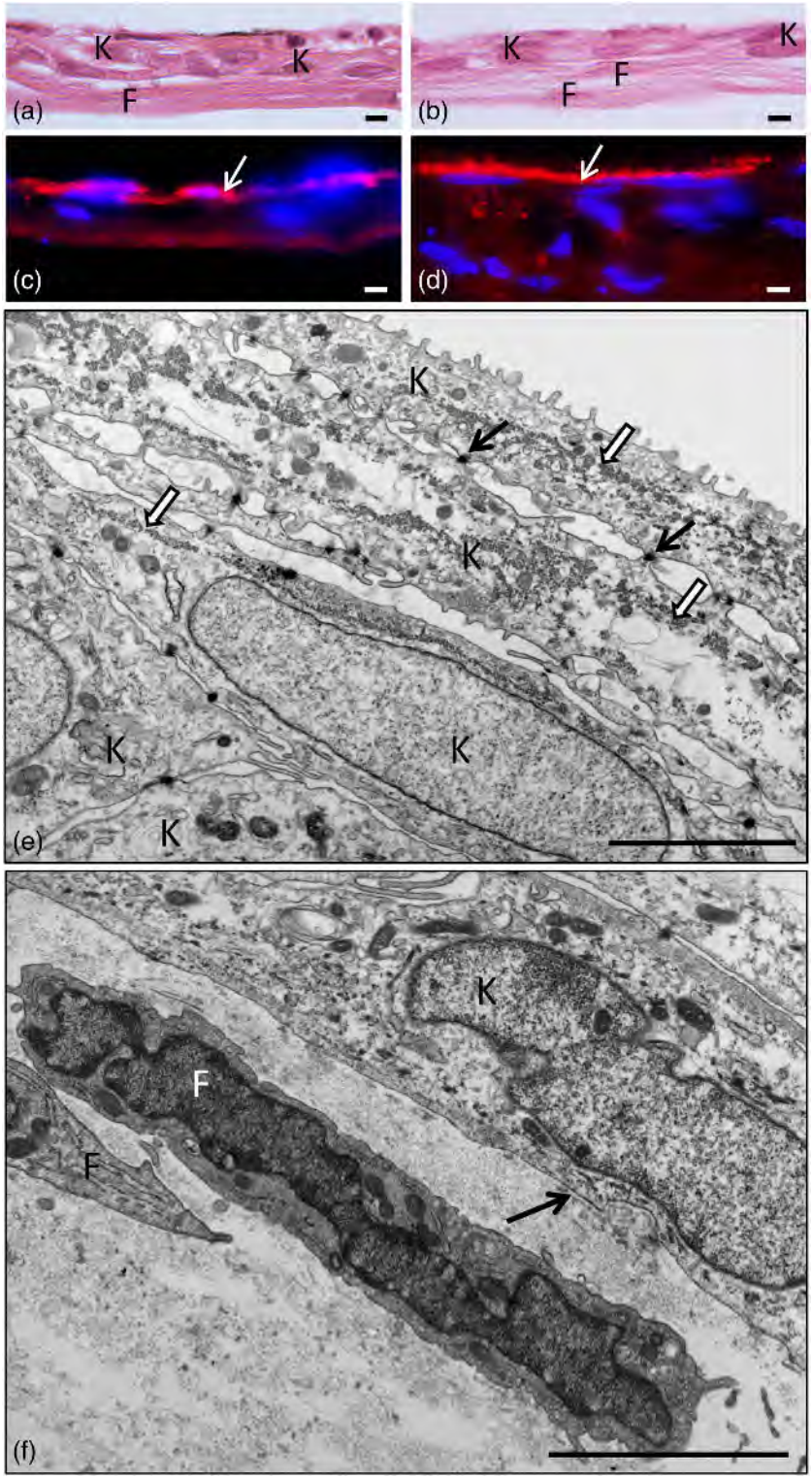

Figure 2. (a, b) H&E-stained sections of 5 week skin composed of keratinocytes (K) and (a) vector control and (b) V3-transduced fibroblasts (F). (c, d) Immunofluorescent staining of keratin with VM-1 in 5-week skin sheets, showing keratin (arrows, orange/red) in upper keratinocytes in control skin sheets (c) and in V3 skin grown on Endoform Dermal Template™ (d). (e, f) Electron micrographs of V3 skin showing (e) multilayers of keratinocytes (K), linked by desmosome junctions (black arrows) and with upper flattened cells containing keratin deposits (open arrows) and (f) interface between keratinocyte (K) layer and the dermal layer of fibroblasts (F) surrounded by extracellular matrix; a developing basement membrane is evident (arrow). Magnification bars = (a–d) 5 μm, (e, f) 2 μm

Versican (V1) mRNA levels were significantly decreased in the V3 and antisense fibroblast sheets at week 4, more so in the latter (~40% decrease). At week 5, with keratinocytes present, V1 mRNA was reduced in antisense cultures only, but to a lesser extent (~30%) than that at 4 weeks. Thus, the knockdown of versican was relatively inefficient. Keratinocytes expressed V1 message at levels similar to the fibroblasts (Figure 5).

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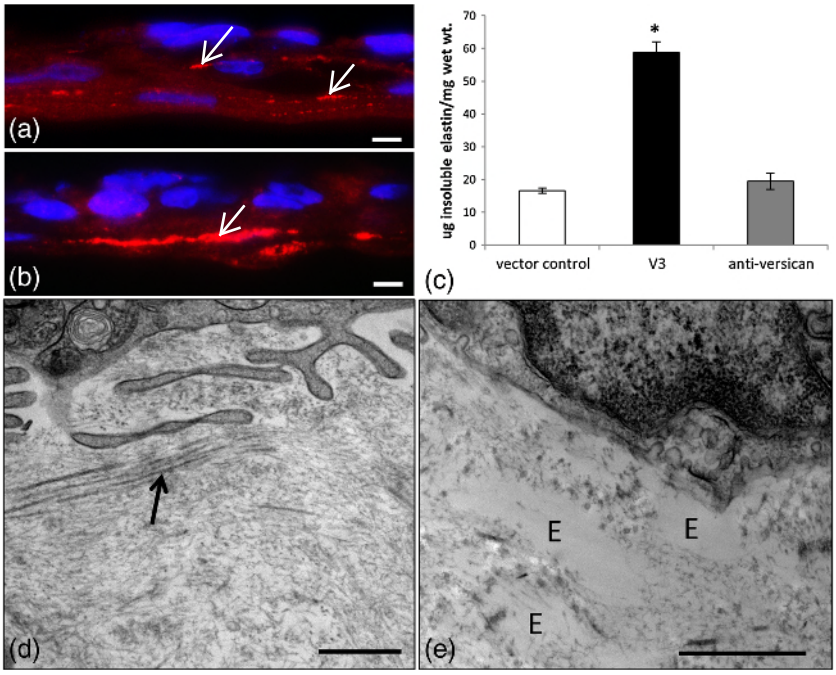

Figure 3. (a, b) Immunofluorescent staining for elastin (arrows, orange/red) in 5-week vector control (a) and V3 (b) skin sheets, showing small scattered deposits of elastin in the dermis of the vector control and large dense contiguous aggregations of elastin in the dermis of V3 skin. (c) Fastin™ assay for extracted insoluble elastin in 4-week vector control, V3 and anti-versican skin sheets (*$p < 0.05$ compared to vector control). (d, e) Electron micrographs of 5-week dermis showing (d) extracellular matrix containing small-diameter woven collagen fibrils (arrow) in a vector control and (e) deposits of elastin (E) in V3 dermis, close to the cell surface on the underside of a fibroblast and amongst collagen fibrils. Magnification bars = (a, b) 5 μm, (d, e) 0.5 μm

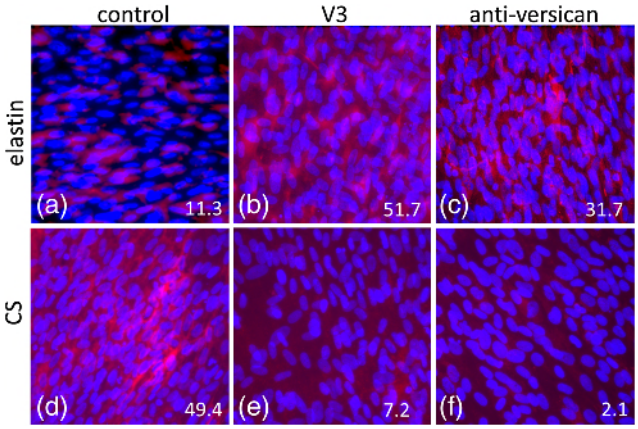

Figure 4. Immunofluorescent staining (orange/red) of 4-week cultures of dermal fibroblasts for elastin (a–c) and chondroitin sulphate (CS; d–f) in vector control, V3 and anti-versican transduced cells, showing reciprocal relationship between elastin and CS staining; number in lower right of each panel is percentage area stained orange/red, determined by ImageJ analysis

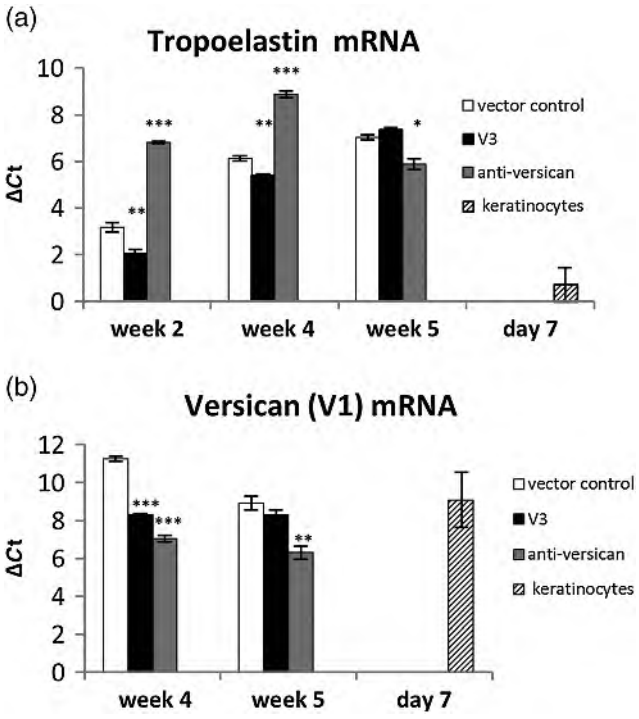

Figure 5. (a) mRNA levels for tropoelastin, determined by qRT–PCR, in 2- and 4-week cultures of fibroblasts, in 5-week cultures of fibroblasts (for 4 weeks) plus keratinocytes (for 1 week) and in keratinocytes cultured alone for 7 days. (b) mRNA levels for versican in 4- and 5-week cultures and in keratinocytes cultured alone for 7 days (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vector control)

3.5. Mechanical strength of skin sheets

Skin sheets were tested for mechanical integrity on a Hysitron TI-950 TriboIndenter. Stress–strain data for vector control and V3 skin sheets are shown in Figure 6a. Compared to vector control skin, V3 skin exhibited increased stiffness, with reduced displacement, in the mid- to upper range of

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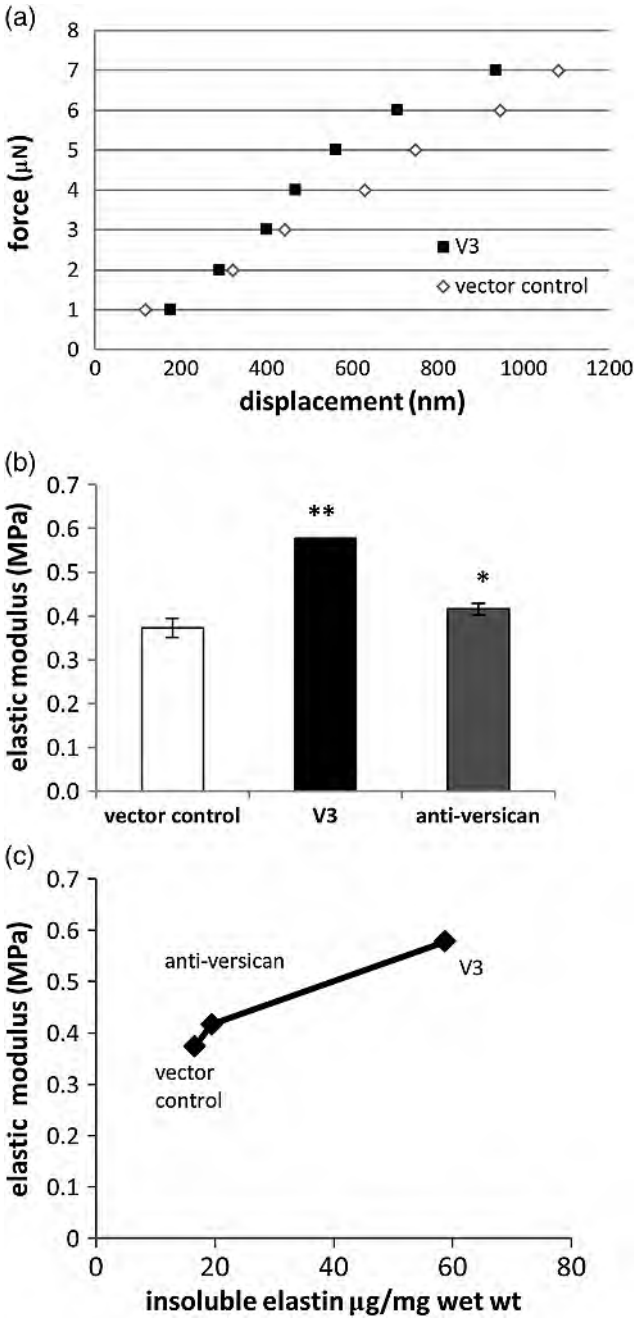

Figure 6. (a) Stress–strain relationship for vector control and V3 skin sheets. (b) Elastic modulus (MPa) for skin sheets at 6 μN. (c) Relationship between elastic modulus and insoluble elastin content of vector control, V3 and anti-versican skin sheets (*$p < 0.05$, ** $p < 0.01$ compared to vector control)

force applied. The thinner anti-versican sheets also showed increased stiffness, but to a lesser degree, with an elastic modulus of 0.42 MPa compared with vector control (0.37 MPa) and V3 (0.58 MPa) at 6 μN (Figure 6b). The elastic modulus was proportionally related to the insoluble elastin content of the skin sheets (Figure 6c).

3.6. Growth of skin sheets on Endoform Dermal Template™

While 5 weeks was sufficient time to produce an elastin-enriched dermis with enhanced mechanical properties, the sheets were thin and difficult to manipulate with instruments once freed from the culture dish substrate. As a first step in circumventing this problem, skin sheets were cultured on the biodegradable substrate Endoform Dermal Template™, a potential carrier for transferring skin sheets from culture dishes to a wound bed. Endoform, however, has been reported to contain sulphated GAGs (Lun *et al.*, 2010), potential inhibitors of elastic fibre formation.

Dermal fibroblasts were seeded onto 2 × 2 cm Endoform sheets, held by plastic frames and immersed in growth medium (Figure 7a–c). The same multiple-seeding protocol was adopted as for culture on plastic, with keratinocytes seeded at week 4 for the final week. Cells were seeded on the adluminal surface and formed a dermis. Keratinocytes formed an epidermal layer (Figure 7d–f) similar to sheets cultured on plastic and, as shown in Figure 2d, were similarly differentiated and contained keratin; 5-week cultures were immunostained for elastin (Figure 7g, h) and elastin content for the dermis, determined by point counting, expressed as percentage volume fraction (Figure 7i). V3 dermis contained a significantly higher content of elastin than vector control dermis. The elastin content of anti-versican dermis was also increased, but not significantly.

4. Discussion

This study demonstrates that overexpression by human dermal fibroblasts of the gene for versican variant V3 is an effective strategy for increasing elastin in the dermis of cultured skin. Unlike extracellular matrix components of collagen and matrix proteoglycans, elastin is deficient in cultured skin substitutes used for grafting and is similarly deficient in scar tissues (Rnjak *et al.*, 2011). The ability to alter the composition of the extracellular matrix in skin cultured *ex vivo* provides an approach to engineering skin that may provide improved outcomes for cultured skin grafts.

Previous studies have demonstrated that overexpression of V3 promotes elastin deposition in matrix laid down by cultured cells, including dermal fibroblasts from patients with Costello syndrome who have a deficiency of elastic fibres (Hinek *et al.*, 2004) and *in vivo* in neointima of vessels formed by cells transduced with V3 (Merrilees *et al.*, 2002, 2011). This study expands on those findings by demonstrating that an elastin-enriched human dermis, covered by a differentiated epidermis, can be produced under organ-culture conditions and within a time frame (5 weeks) that is short enough to be clinically useful.

Shorter times for culture of a dermo-epidermal skin substitute are possible (Pontiggia *et al.*, 2013) through the use of seeded collagen hydrogels as a starting point for dermal construction, although it is unclear whether shortened culture periods of < 2 weeks allow for the development of fibroblast-derived extracellular matrix with formed components. Our previous investigations (Merrilees *et al.*, 2002) and the results of this present study indicate that collagen bundles and elastic fibres take > 2 weeks to form.

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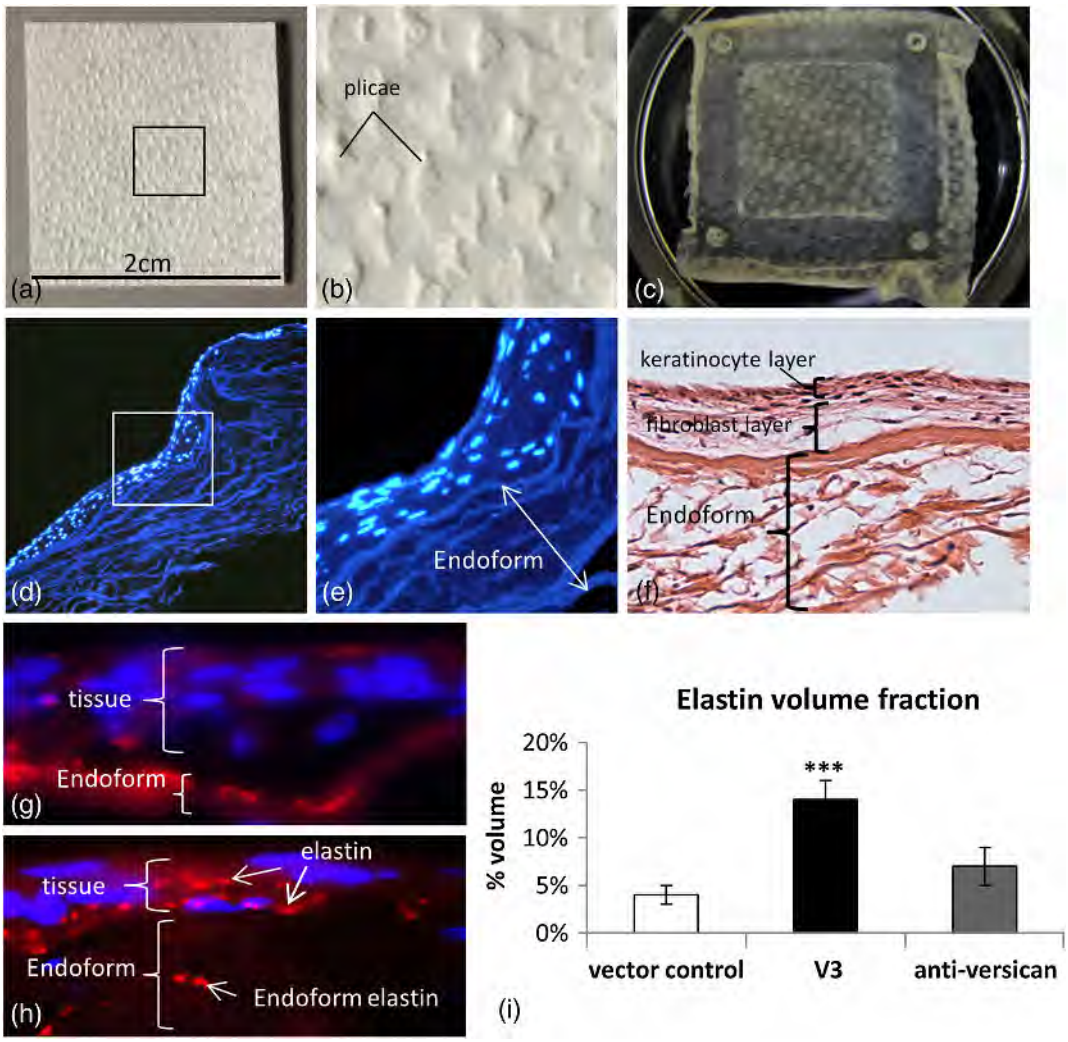

Figure 7. Growth of skin sheets on Endoform Dermal Template™. (a) Endoform sheet, prepared from submucosal layer of ovine forestomach, showing adluminal surface with plicate folds, enlarged in (b). (c) Cultured skin (5 weeks) on Endoform sheet. Position of holding frames marked by square area devoid of cells. (d–f) Histological sections of Endoform, showing 5-week cultured skin on adluminal surface, stained with DAPI (d) with boxed area at higher magnification (e) and H&E (f). (g, h) Vector control (g) and V3 (h) 5-week skin on Endoform, immunofluorescently stained for elastin (orange/red), with ovine elastin present in Endoform template. (i) Elastin content, measured by morphometry and expressed as a volume fraction percentage of the dermal layer, for vector control, V3 and anti-versican 5-week skin sheets cultured on Endoform (*$p < 0.001$ compared to vector control)**

Preliminary investigations showed that a single seeding of fibroblasts, while capable of producing a multilayered dermis over 4 weeks, produced thinner sheets than the strategy of multiple fibroblast seedings, which resulted in thicker sheets, more easily manipulated. The strategy of not introducing keratinocytes until the end of week 4 of culture, after the dermal layer was established, was designed to allow for the formation of a dermis and elastin deposition before possible negative influences from the keratinocytes. Notably, we found that the level of V1 expression was similar to fibroblast expression, and this additional versican could negatively impact elastin deposition in the adjacent dermis. Subjectively, the elastin in our skin sheets was predominately located in the lower dermis. It has been reported previously that cultured keratinocytes express versican mRNA and that versican is present in the stratum basale of the epidermis (Zimmermann *et al*., 1994), and later studies have established that CS of versican (Huang *et al*., 2006) and biglycan (Hwang *et al*., 2008) negatively influence the EPB-mediated transfer of tropoelastin to growing elastic fibres at the cell surface. While the influence of epidermal-derived versican on elastin deposition in the dermis remains to be determined, our protocol of first establishing a dermis prior to the plating of keratinocytes proved to be a successful strategy. Nevertheless, long-term outcomes for skin sheets *in vivo* are not yet known.

As reported previously, elastic fibres form at the cell surface. In culture, fibres are deposited on the undersides of cells (Merrilees *et al*., 2002). A similar pattern of deposition was observed in the skin sheets, with small, isolated but aligned deposits in the vector control sheets and aggregations of larger and contiguous deposits in the V3, and to a lesser extent anti-versican, sheets. Deposition and assembly were similar in all sheets, but occurred to a greater degree in the overexpressing

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sheets.The amount of elastin deposited in response to anti-versican overexpression, however, was less than seen for another model system, cultured vascular smooth muscle cells overexpressing the same antisense sequence *in vitro* and *in vivo* (Huang *et al*., 2006). The reason for the comparatively small effect on elastin in the skin sheets is likely the modest knockdown of versican expression. A higher level of knockdown is associated with markedly increased elastin deposition (Huang *et al*., 2006). We do not have data, however, on the titration of versican levels or expression against elastin deposition. Further, the relationship is difficult to dissect, as it is the distribution of versican that appears critical, with a reduction in pericellular CS favouring EBP-mediated fibre assembly.

Tropoelastin mRNA levels generally did not correlate with measures of insoluble elastin or with amounts of elastin deposited in the dermal layer, although notably message levels were increased in the 2- and 4-week skin sheets expressing versican antisense. In the presence of keratinocytes, however, levels for all groups were similar. While some studies have reported a positive correlation between tropoelastin message levels and insoluble elastin (Huang *et al*., 2006; Merrilees *et al*., 2002), others have shown that marked elevation in tropoelastin expression can occur without the deposition of elastin. Balloon-induced neointimal thickenings in vessels show a marked increase in message without the deposition of elastin (Nikkari *et al*., 2004), as do alveolar margins in lungs with emphysema (Lucey *et al*., 1998), observations that, together with the results of this current study, indicate that synthesis and assembly of elastin need not be linked.

Subjective assessment of the strength of skin sheets manipulated by surgical instruments indicated that the V3 sheets were stronger than the vector control sheets. Ultrastructurally, the extracellular matrix of the V3 sheets appeared more compact. These assessments were confirmed by mechanical testing data that showed that V3 skin sheets were stiffer, with more resistance to deformation, than vector control sheets and with a higher elastic modulus. The anti-versican sheets were marginally stiffer than the control sheets. The elastic modulus, however, was low compared to normal skin, which has a wide range of values (~50–90 MPa) (Krueger *et al*., 2011; Ní Annaidh *et al*., 2010), depending on location. A more appropriate comparison would be with human fetal skin from very early development, but such values do not appear to have been calculated. Amniotic membrane at term, however, has an elastic modulus of 2.3 MPa (Niknejad *et al*., 2008), a value more similar to our skin sheets.

While elastin may not be the sole determinant of the increase in stiffness, vessels cultured from smooth muscle cells overexpressing V3 exhibit greater structural integrity and a higher resistance to burst than vector control equivalents at strains > 12% (Keire *et al*., 2010). A caveat on interpretation of cultured vessel strength is that ascorbate levels modulate stiffness (Keire *et al*., 2010) and *in vivo* testing will be required to determine whether cultured skin with an increased elastin content and increased stiffness results in an improved outcome after grafting.

Production of autologous skin sheets in the shortest possible time is important clinically. The 5 weeks taken to produce our bilayer skin sheets is within an acceptable time frame, but with the disadvantage that the sheets are thin and difficult to manipulate, and thus are likely less than suitable for covering a wound bed. In a first attempt to overcome this problem, we report here on the growth of skin sheets on a recently approved biodegradable wound dressing, Endoform Dermal Template™, to provide a potential carrier, as opposed to an integrated scaffold, for cultured skin. Initial results are encouraging, with bilayered skin sheets cultured on the 'luminal' surface of the Endoform and with the reported preservation in Endoform of CS moieties (Lun *et al*., 2010) not inhibiting the deposition of elastin in the dermal layer. It remains to be determined, however, whether degradation of Endoform, likely associated with a versican-rich inflammatory matrix, compromises the increased elastin content of the young skin. Interestingly, forced expression of V3 may confer anti-inflammatory properties on tissues. *In vivo*, V3 expression by vascular smooth muscle cells reduces the ingress of monocytes into vessel walls, and *in vitro* the adhesion of monocytes to V3-generated matrix is reduced (Merrilees *et al*., 2011).

Finally, our results confirm that forced expression of V3 is an effective tool for promoting elastin deposition. There remain, however, unresolved issues surrounding gene therapy. This may be circumvented by protein-based treatment of skin sheets with V3 but, equally, recent developments on improved adeno-associated virus vectors (Mingozzi and High, 2013; Sen *et al*., 2013) point to therapeutic applications, and our *ex vivo* approach may be especially suitable in this regard.

In summary, we have produced, over a 5-week period of culture, bilayered human skin sheets with a dermis that contains an increased content of elastin and an epidermis composed of layers of keratinocytes that undergo differentiation. The protocol to achieve these features involved prior construction of the dermal layer, to ensure initial formation of an elastin-rich matrix, before the introduction of keratinocytes that secrete the elastin inhibitor versican. The long-term stability and usefulness of the elastin-enriched skin constructs *in vivo* remains to be determined.

Conflict of interest

B. May is a shareholder in Mesynthes Ltd.

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