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# Endoplasmic reticulum stress in human chronic wound healing: Rescue by 4-phenylbutyrate

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**Abstract**

During wound healing, cells have a high rate of protein synthesis and many proteins need to be folded post-translationally to function, which occurs in the endoplasmic reticulum (ER). In addition to proliferation, several cellular stress conditions, such as hypoxia, in the wound micro-environment lead to the accumulation of unfolded or misfolded proteins in the ER, causing ER stress. Eukaryotic cells have a signalling system to manage ER stress called the unfolded protein response (UPR). Mild UPR activation has a beneficial homeostatic effect; however, excessive UPR induces cell death. Herein, we examined venous leg ulcer biopsies versus normal acute incisional wounds in age-matched elderly subjects and found a large increase in ER stress markers. To study the underlying mechanism, we established several cell cultures from amputated legs from the elderly that showed inherent ER stress. While both keratinocytes and fibroblasts migration was impaired by ER stress, migration of elderly leg skin keratinocytes was markedly improved after treatment with the chemical chaperone and clinically established drug 4-phenylbutyrate (4-PBA) and demonstrated a reduction in ER stress markers. In a full-thickness human skin wound healing model, 4-PBA improved the reepithelialisation rate, which suggests it as a promising drug repurposing candidate for wound healing.

**KEYWORDS**

chronic leg ulcers, drug repurposing, ER stress, skin, wound healing

## 1 | INTRODUCTION

### 1.1 | Chronic leg ulcers

Wound-related costs are estimated to about 2% to 4% of health care budgets in the industrialised world, and this is expected to increase with an ageing population.[1] Chronic wounds most often appear on the legs and the common primary causes include: venous insufficiency (50%-60%), arterial insufficiency (15%-20%), and diabetes (5%).[1] There is a major need for research aiming to develop new robust therapies for chronic leg ulcers.

Importantly, because of the complexity of chronic wounds, targeting central cellular pathways is more likely to be successful than narrow mechanisms. Another important aspect is that repurposing of already approved drugs is financially much more realistic than developing novel compounds for this patient category.

### 1.2 | Unfolded protein response and endoplasmic reticulum stress

The endoplasmic reticulum (ER) performs multiple essential cellular tasks, including lipid synthesis, protein folding and calcium storage. To gain proper function, many proteins need to be post-translationally folded into specific 3D structures.[2] Several cellular stress conditions that lead to the accumulation of unfolded or misfolded proteins in the ER lumen are collectively called "ER stress." The unfolded protein response (UPR) is the system that eukaryotic cells have developed to overcome cellular damage induced by ER stress. In mammals, three ER stress transducers, namely protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring 1α (IRE1α), and activating transcription factor 6 (ATF6), play key roles in the UPR signal transduction. The homeostatic impact of the UPR is played out on several levels, including increased ER protein folding capacity and reduction of protein load.[3] A pathological role of ER stress has been established in several human pathologies,[4] however, to date ER stress has not been examined in human wound healing.

### 1.3 | Wound healing and ER stress

The general course of wound healing is divided into the inflammatory, the proliferative (neoangiogenesis, tissue formation, reepithelialisation) and the tissue remodelling phases. ER stress has been associated with several of the basic processes involved in these phases. For example, ER stress regulates inflammation[5] and is important in proliferating cells because of its central role in folding newly synthesised proteins required to build new cells.[6] In cancer, ER stress has been linked to cell migration,[7] which is a central part of wound healing. With this in mind, we hypothesised that ER stress may be a plausible target in clinically relevant human wound healing.

In this translational study, we have for the first time examined ER stress in chronic human venous ulcers compared with normal age and anatomic site-matched wound healing. We further examined the effect of clinically established ER stress relieving agents on human in vitro and ex vivo wound healing models.

**Key Messages**

- chronic leg ulcers are a tremendous problem in the elderly
- this study is the first to characterise cell stress in human wound healing and identifies a drug that is currently used for other diseases that improves wound healing and thus may be suitable for treating hard-to-heal human ulcers

## 2 | RESULTS

### 2.1 | ER stress in normal lower leg skin wound healing versus venous ulcers

To characterise ER stress in human wound healing, we first examined the expression of the canonical marker spliced *XBP1* (*XBP1s*), which is downstream of IRE1α. We used samples from chronic venous ulcers in elderly patients and as controls age and donor site matched skin biopsies from normal acute wounds of the lower legs (Figure 1A,B). Thus, we were able to characterise both normal, as well as, chronic impaired wound healing in the legs of the elderly. *XBP1s* increased in normal wound healing at 7 days post incision with a similar increase observed in venous ulcers; A 1.86 and a 1.77-fold change, respectively, as compared with 1 day post incision (Figure 1C). Three key UPR proteins, including XBP1s, CHOP, and ERO1α were examined using western blotting and showed a similar time dependent increase matching the *XBP1s* expression (Figure 1D). Based on these findings, we also examined broad UPR relevant gene expression by the nCounter gene expression assay[8] in which we compared normal wounds to venous ulcers (Figure 1E). Our analysis focused on the differences between ulcer types (normal acute incisional wounds vs chronic venous leg ulcers), and raw gene expression, including intact skin are shown elsewhere (Figure S1). We examined the expression pattern of ER resident genes, which are involved in the protein-folding process in the ER and UPR, as well as degradation machinery and general cell stress and apoptosis-related genes. We compared the expression of these genes in chronic

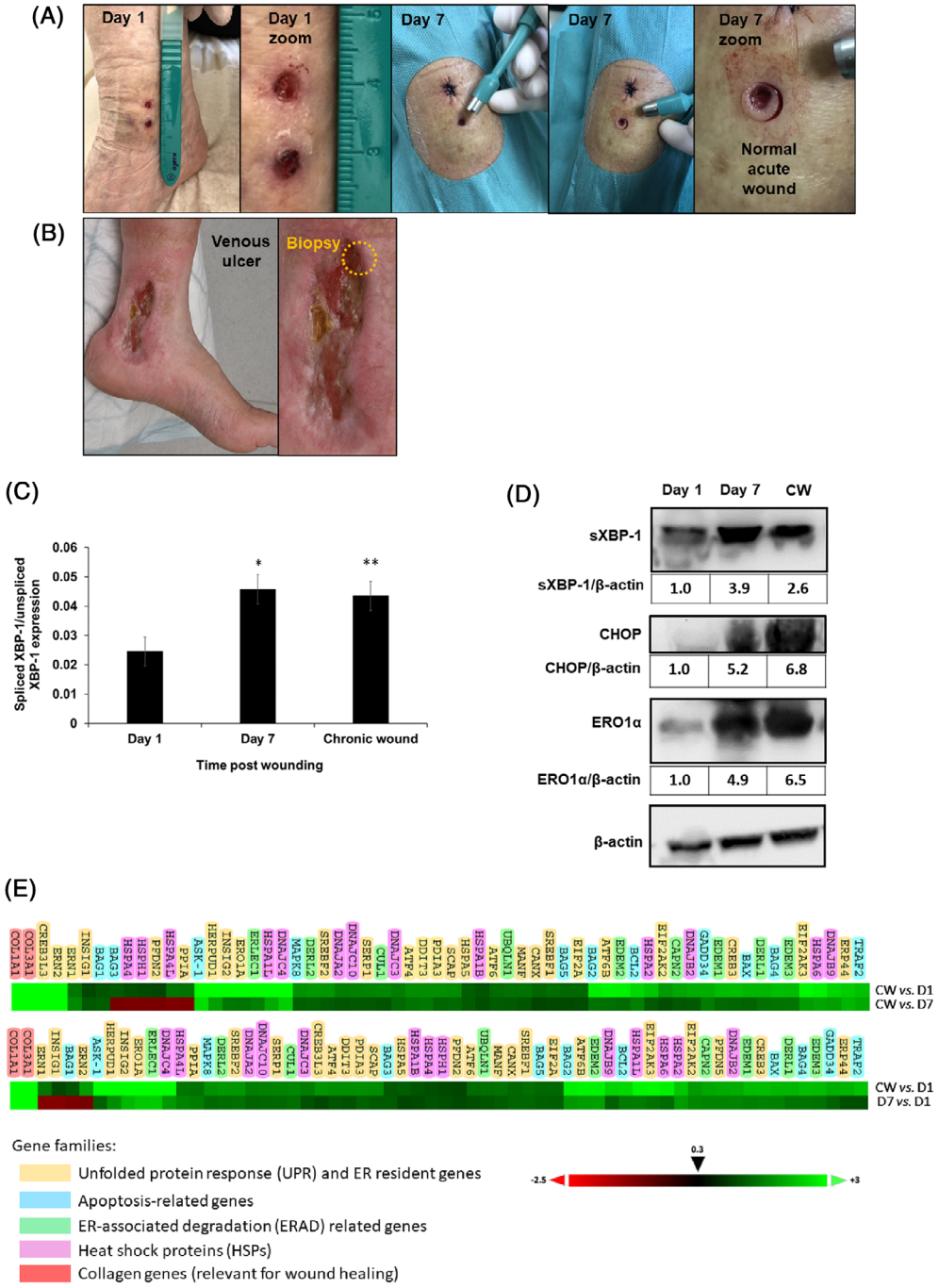


**FIGURE 1** Endoplasmic reticulum (ER) stress is increased in chronic venous ulcers and acute normal wounds. A, Biopsy technique of human lower leg skin. Example of healthy volunteer 60+ years old, in which two 4 mm punch biopsies were taken thus creating two wounds, followed by one of the wounds being excised with a 6 mm punch biopsy knife on day 1, and this was repeated 7 days post incision. This generates skin samples of acute normal wounds at different time points. B, Biopsy technique of chronic venous leg ulcer. Example of 60 + years patient in which the biopsy was taken at the wound edge as indicated by the orange circle. C, Spliced *XBP-1* expression analysed by qPCR in acute leg wounds 1/7 days post incision and chronic venous leg ulcers (n = 7 and n = 11, respectively). Data are normalised to 18S and presented as a ratio between spliced *XBP-1* to unspliced *XBP-1*. D, Western blot analysis of the key ER stress markers CHOP, ERO1α, XBP-1s in acute leg wounds after 1/7 days and in chronic venous leg ulcers. Densitometric quantifications corrected to β-actin shown below the western blots, normalised to day 1 samples (1.0). E, Gene expression analysis by nanoString nCounter of multiple genes primarily related to unfolded protein response and ER stress as well as genes related to apoptosis (since ER stress can induce apoptosis) and collagen (control for biopsy quality, upregulated in wounds). mRNA expression values were normalised to two stable housekeeping genes in each sample, that is, each samples' gene expression was normalised to its housekeeping genes. Relative gene expression shown. Note that CW versus D1 appears twice (but in different gene order) because of limitations of the analysis software. All biopsies from subjects ≥60 years. N = 7 for D1 and D7, n = 11 for CW. *$P < .05$, **$P < .01$, ***$P < .001$. CW, chronic wound (chronic venous leg ulcer)

wounds versus acute incisional wounds after 1 (D1) and 7 days (D7), and found increased expression in chronic venous ulcers (CW). We discerned: (a) Upregulation of heat shock proteins (HSP) genes, which are associated with activation of the UPR machinery. (b) Upregulation of UPR genes such as *ERN2*, *ATF6b*, and *ATF4* and downstream targets *GADD34* and *PERK*. (c) A pronounced increase in the ER oxireductase *ERO1α* that plays a role in ER stress-induced CHOP-dependent apoptosis[9] (3.72, 1.53, and 2.43 fold more in CW vs D1 and CW vs D7, and D7 vs D1, respectively; Table S2), and *PDI* and *ERp44*, which act downstream of the IRE1a-XBP1s-CHOP arm, and facilitates oxidative protein folding in the ER as well.[10] (d) Upregulation of ER-associated degradation (ERAD) related genes downstream of ATF6 and XBP1s such as *EDEM2* and *ERLEC1* aiming to eliminate misfolded proteins. (e) Upregulation of stress and apoptosis-related genes such as *TRAF2* and *ASK1* acting downstream of IRE1α[11] (2.35 and 2.9 fold and 3.5 and 2.7 fold more in CW vs D1 and CW vs D7, respectively), which suggests that ER stress may have caused cell death in the wounds. Of note, we found increased expression of *COL1A1* and *COL3A1* in CW versus acute incisional wounds with a decreased type 1/type 3 collagen ratio, which is typical of the early wound healing phases that chronic wounds are unable to proceed from.[12] Overall, ER stress related gene expression was upregulated in venous ulcers compared with day 1 or day 7 after incision in normal wound healing, and ER stress was also higher in day 7 as compared with day 1.

## 2.2 | ER stress impairs cell migration and wound healing in keratinocytes and fibroblasts

To examine whether ER stress per se can be of any pathophysiological significance in the skin's main cell types, we tested how it affects one of the most basic wound healing processes: cell migration. First, the classical ER stress inducing drugs tunicamycin that inhibits ER protein glycosylation, and thapsigargin that depletes ER calcium ($Ca^{2+}$) by sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibition, were titrated on human dermal fibroblasts and keratinocytes using the Incucyte automated scratch assay (Figures 2A,B and S2-S4). Tunicamycin and thapsigargin clearly impaired scratch assay wound closure at relatively low concentrations in both cell types. During the first 24 hours of scratch assays, cell migration dominates over proliferation and thus the ER stressors appeared to inhibit migration primarily because of their almost immediate effect; this, however, was confirmed by using the proliferation inhibitor mitomycin C, which only had a minor effect in keratinocytes (Figure 3). Thus, ER stress primarily impaired cell migration.

## 2.3 | Chemical chaperones rescues ER stress inherent impairment of cell migration in keratinocytes but not fibroblasts

As pharmacologically induced ER stress impaired migration of both fibroblasts and keratinocytes, we tested if such stress could be rescued by treatment with the chemical chaperones 4-PBA or TUDCA in both cell types (Figure 2A-G). For this purpose, we established several fibroblasts and keratinocytes cultures derived from lower leg skin from elderly leg amputation donors, as well as, from truncal skin of younger plastic surgery donors. This enabled us to examine cells both similar and dissimilar to those in chronic venous ulcers; that is, the cells were matched by age, donor site, and general disease. Importantly, we found that elderly leg keratinocytes migrate much more slowly than young truncal keratinocytes with 10% to 40% compared with 80% to 100% healing, respectively, after 72 hours, which indicates major impairment in wound healing capability. A dose titration of chaperones in both young truncal and elderly leg fibroblasts showed no rescue effect (Figures 2A,B and 3A,B) and a similar result was found in young truncal keratinocytes (Figure 2C). However, in keratinocytes from elderly leg amputation donors the chaperones TUDCA and 4-PBA had a substantial rescue effect, the latter being larger (Figure 2D-G). Thus, as expected, fibroblasts and keratinocytes are both sensitive to ER stress, but differ in their chaperone sensitivity.

## 2.4 | ER dilation in wound keratinocytes

As the wound biopsies used for gene expression and western blot were of mixed cell types, we searched for evidence that ER stress was indeed present in keratinocytes. ER dilation was shown to be associated with ER stress in several studies.[13] By transmission electron microscopy, we identified cells adjacent to, as well as, far from the wound bed, respectively, in both day 7 normal wounds and chronic venous ulcers (Figure 4A). ER dilation was clearly present in keratinocytes adjacent to the wound bed but not far from the wound bed (Figure 4B-E). This suggests that ER stress in keratinocytes is linked to the wound reepithelialisation process.

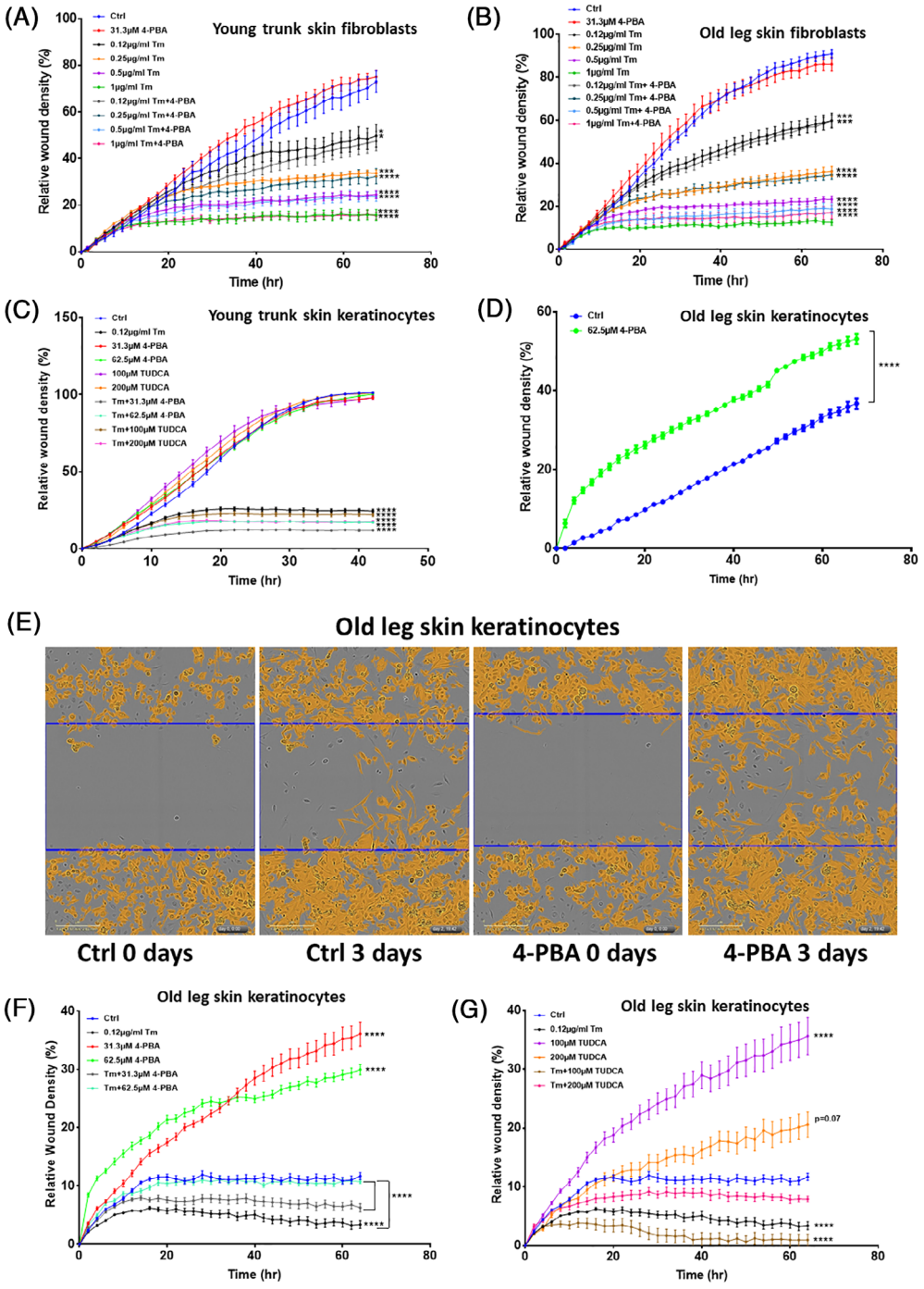


**FIGURE 2** Endoplasmic reticulum (ER) stress impairs cell migration in fibroblasts and keratinocytes while chemical chaperones improve cellular migration only in elderly skin keratinocytes. Scratch wound assay of fibroblasts and keratinocytes derived from both young and aged skin and treated with different pharmacological ER stressors and chemical chaperones. A, Young trunk skin fibroblasts ± Tm and 4-PBA for 48 to 72 hours. B, Old leg skin fibroblasts ± Tm and 4-PBA for 48 to 72 hours. C, Young trunk skin keratinocytes ± Tm, 4-PBA and TUDCA for 48 hours. D, Elderly skin keratinocytes ± Tm and 4-PBA for 72 hours. E, Representative images of elderly skin keratinocytes ± 62.5 μM 4-PBA for 72 hours (yellow scale bar 300 μm). (F) Elderly skin keratinocytes ± 4-PBA and TUDCA for 12 hours pre-scratching followed by ± Tm, 4-PBA and TUDCA for 48 hours post-scratching. N = 3 with mean ± SE. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$ compared with ctrl unless indicated otherwise by brackets, by two way-analysis of variance (ANOVA). 4-PBA, 4-phenylbutyrate; Ctrl, control; Tm, tunicamycin

## 2.5 | Reversal of inherent ER stress in elderly leg keratinocytes by chemical chaperones

To understand the mechanism behind how the chemical chaperones impacted the UPR, we examined a set of central UPR makers by RT-quantitative reverse transcription PCR (qPCR) in cultured keratinocytes (Figure 5). In accordance with previous results showing a reduced healing rate in elderly leg keratinocytes compared with young truncal cells (Figure 2D-G), we observed a marked increase in the mRNA expression of *ATF4*, *ATF6β*, *CHOP*, *GRP78*, *XBP1s*, *ERO1α* (24 and 48 hours), and *eIF2α* (48 hours) (and a trend of increase in *ERN1*) in untreated elderly leg cells. After 24 and 48 hours—the expression of these markers was significantly reduced by 4-PBA, demonstrating alleviation of ER stress. Importantly, the rescue effect of 4-PBA was not observed in young truncal keratinocytes. To verify that the effect of 4-PBA was ER stress-related, we used another chemical

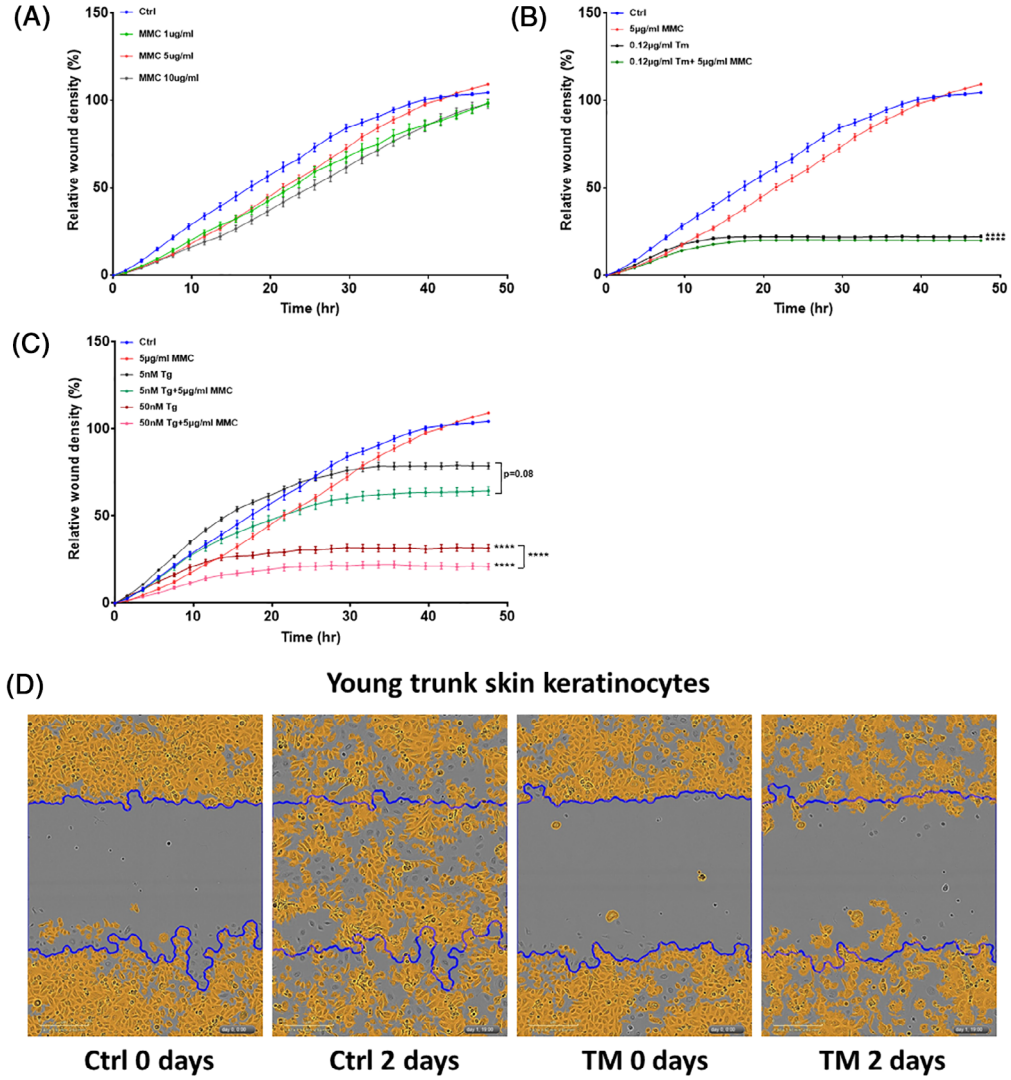


**FIGURE 3** Endoplasmic reticulum (ER) stress primarily impairs keratinocyte migration and not proliferation. Young trunk skin keratinocytes. A, ± MMC 1 hour pre-scratching. B, ± MMC 1 hour pre-scratching followed by ± Tm 48 hours post-scratching. C, ± MMC 1 hour pre-scratching followed by ± Tg 48 hours post-scratching. D, Representative images (yellow scale bar 300 μm). N = 3 with mean ± SE. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$ compared with ctrl, unless indicated otherwise by brackets, by two way-analysis of variance (ANOVA). Ctrl, control; MMC, mitomycin C; Tm, tunicamycin; Tg, thapsigargin

chaperone—TUDCA. After 24 to 48 hours TUDCA treatment, the mRNA expression of the majority of the markers was decreased thus further supporting the existence of elevated ER stress in elderly but not young keratinocytes. To ensure that the chaperone concentrations were adequate, we verified that they were able to rescue from chemically induced ER stress by tunicamycin in young truncal keratinocytes. In contrast, treating elderly leg keratinocytes with tunicamycin induced only a minor increase in ER stress markers (*ATF4* $P = .06$, *CHOP* $P = .06$ [24 hours], and <.05 [48 hours]), thus suggestive of a highly saturated stress state, which could be rescued by 4-PBA but to a lesser extent (compare to the massive rescue effect on the control leg cells) as depicted by reduced expression of the ER-stress markers. Thus, the elderly leg skin keratinocytes showed inherent basal ER stress but, on the other hand, were available for chaperone rescue.

## 2.6 | Topical 4-PBA improves reepithelialisation of full thickness human skin wounds

As 4-PBA was most efficient in improving cell migration and reducing ER stress in keratinocyte cultures, we examined its effect on human skin wounds using the ex vivo human skin wound healing model[14] (Figure 6). We opted for this human ex vivo model as animal wound healing models are strikingly different from human.[17] The skin biopsies were harvested from truncal skin from young plastic surgery patients as the skin biopsies from amputated legs became necrotic in culture. It is important to understand, however, that the ex vivo human skin wound healing model is hypoxic because of the absence of blood supply and diffusion limitations of oxygen and is thus a suitable model for ER stress, which is induced by hypoxia.[18] 4-PBA treatment led to faster

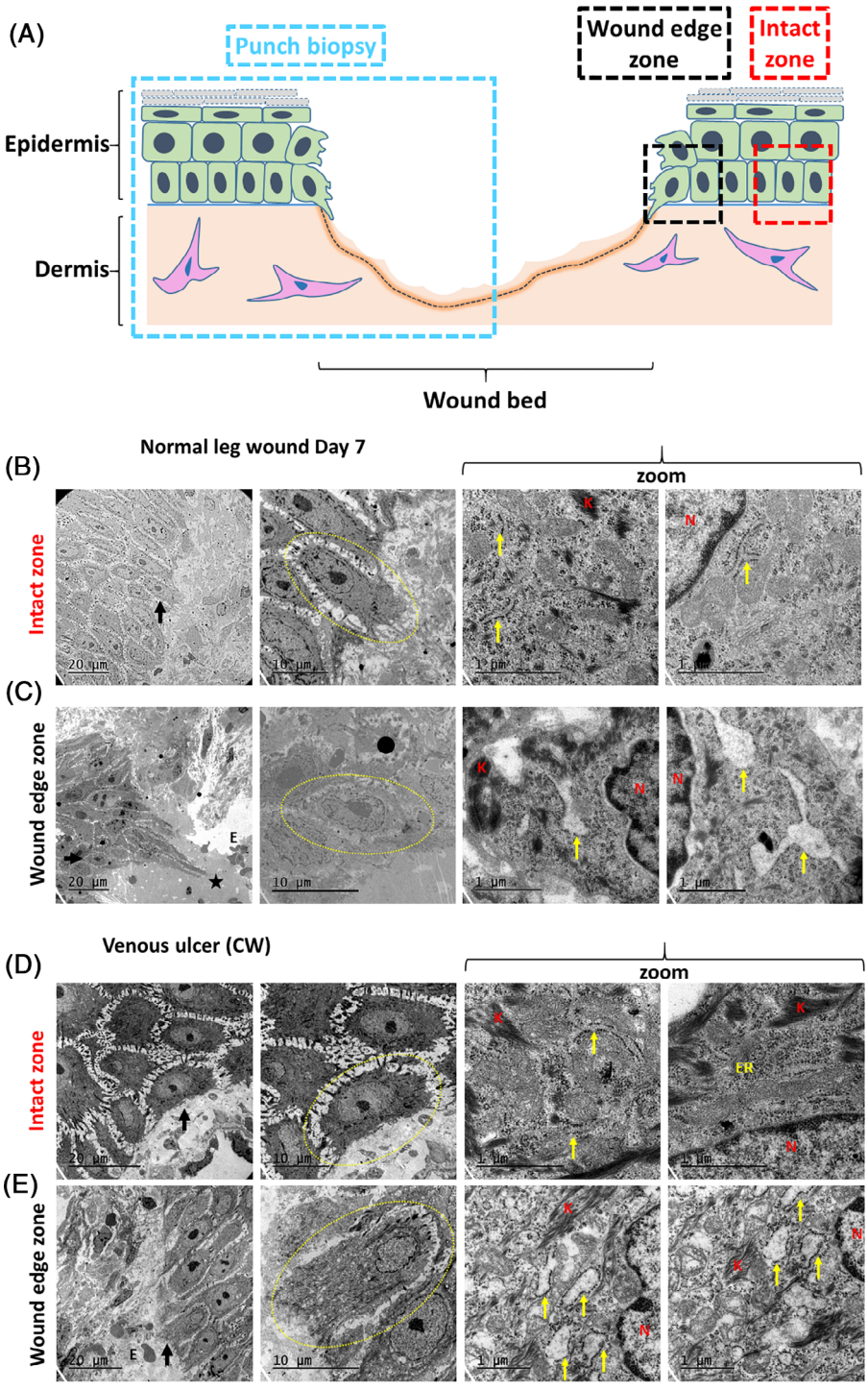


**FIGURE 4** The endoplasmic reticulum (ER) is dilated in keratinocytes adjacent to the wound edge. A, Cartoon illustrating transmission electron microscopy imaging of wound edge zone and intact epidermal zone, as well as how punch biopsies were taken (50% intact epidermis, 50% wound bed). B-E, Transmission electron microscopy images. The rough ER is identified by ribosomes (black punctae). Note the ER dilation in the basal layer keratinocytes adjacent to the wound edge zone. ER, endoplasmic reticulum; N, nucleus; K, keratin. Yellow arrows indicate ER. Black arrows and yellow ovals indicate cells imaged. Star indicates migrating epidermal tongue

reepithelialisation with transdermal treatment, in which the drug penetrates the skin by diffusion from the culture media, being slightly superior to topical treatment. Of note, the migrating keratinocytes at the wound edge (epidermal tongue) clearly resemble the in vivo wounds as imaged by electron microscopy (Figure 4C, left image).

## 3 | DISCUSSION

We found evidence of ER stress and upregulated UPR primarily in human chronic venous leg ulcers but also during normal wound healing in the elderly. We further show how ER stress impacts wound healing mechanistically by impairing cell migration and found that the

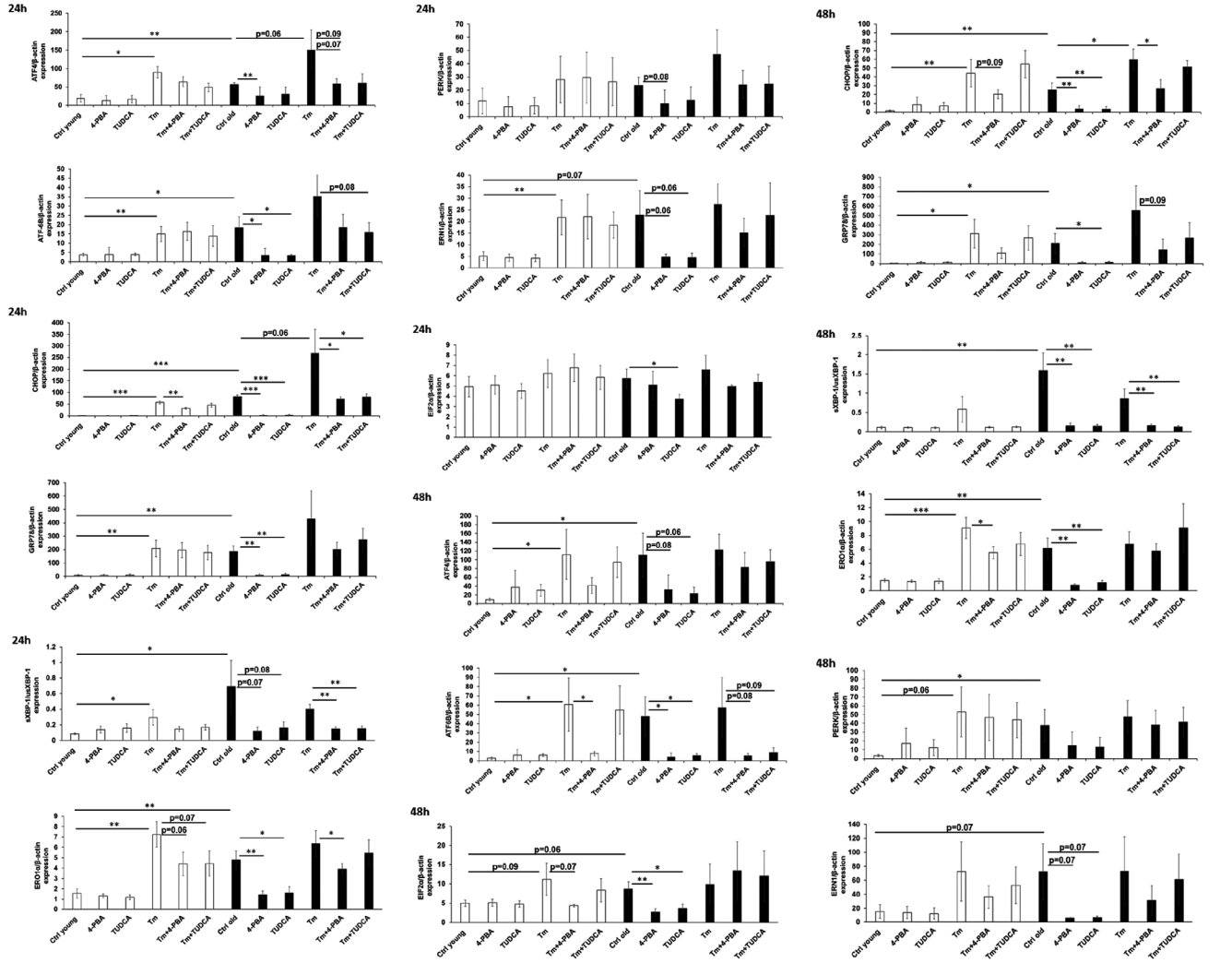

**FIGURE 5** Endoplasmic reticulum (ER) stress is increased in elderly leg skin keratinocytes and 4-PBA alleviates it. CHOP, GRP78, spliced XBP-1, unspliced XBP-1, PERK, ERO1α, ATF6β, ERN1, eIF2α and ATF-4 in primary keratinocytes derived from both young trunk skin and elderly leg skin. mRNA expression levels measured by qRT-PCR and corrected to β-actin following 24 and 48 hours treatments with and without 62.5 μM 4-PBA, 200 μM TUDCA, ± 0.12 μg/mL Tm. For each time point n = 4 with mean ± SE. *$P < .05$, **$P < .01$, ***$P < .001$ by unpaired two-tailed Student's *t* test. Ctrl-control, Tm-tunicamycin, s/unXBP-1-spliced/unspliced XBP-1

chemical chaperone 4-PBA improves migration of aged leg keratinocytes, as well as healing of a human full thickness skin wound model.

## 3.1 | ER stress in wound healing

The wound healing micro-environment is characterised by several factors known to induce ER stress including oxidative stress, hypoxia, infection and nutrient deprivation.[19,20] Mechanistic data on ER stress involvement in wound healing is scarce. Mild ER stress was shown to promote in vitro differentiation of dermal fibroblasts into myofibroblasts, which participate in wound contraction.[21] The few animal studies on ER stress in wound healing show induction of ER stress in diabetic wounds in mice,[22] and a beneficial role of IRE1α signalling for diabetic wound healing.[23] ER stress was also reported in pressure ulcers in rats.[24] However, it is important to note that rodent skin wound healing differs quite substantially from humans.[25] To the best of our knowledge, ER stress has not been investigated in human skin wound healing. We found that several UPR markers as well as ER stress associated genes such as HSPs, ERAD related genes, ER resident chaperones, and apoptosis related genes were upregulated in human chronic venous leg ulcers (Figure 1). As a comparison, we harvested biopsy samples from the lower legs of elderly donors, the most common site of venous ulcers, to examine normal wound healing in the elderly and found a similar UPR

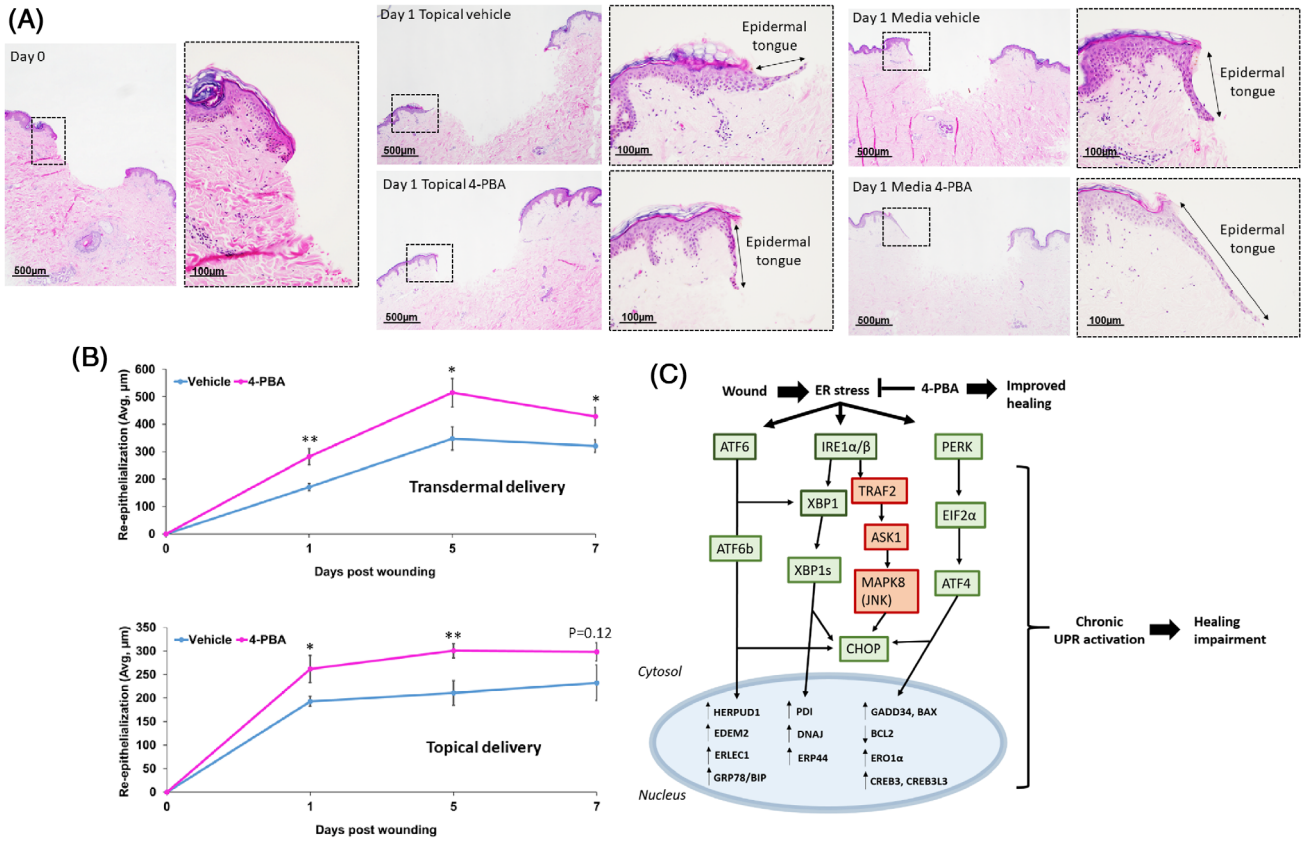


**FIGURE 6** 4-PBA improves wound reepithelialisation and model of endoplasmic reticulum (ER) stress in wound healing. A, Representative images of haematoxylin and eosin staining of human ex vivo full thickness wounds treated with topical or transdermal (media) delivered 4-PBA or vehicle. Black arrows point to representative newly formed epidermal tongues 1 day after incision. ×4 (500 µm) and ×20 objectives used (100 µm). B, Reepithelialisation quantified for day 1, 5 and 7 compared with the initial wound size as shown in day 0. For each time point n ≥ 5 with mean ± SE. *$P < .05$; **$P < .01$; ***$P < .001$ by unpaired two-tailed Student's *t* test. C, Model of ER stress in wound healing. During normal and chronic incision, there is an increase in ER stress followed by activation of the UPR. To restore protein homeostasis, the three classical UPR signalling arms [ATF6, IRE1α/β (ERN1, ERN2-the latter one is selectively expressed in epithelial cells[15]), PERK] are all activated to different levels (green boxes). Transcription factors such as spliced XBP1 (XBP1s), ATF6b, and ATF4[16] migrate to the nucleus to transactivate genes of the ERAD (HERPUD1, EDEM2, ERLEC1), chaperones (BIP) and HSPs (HSPA6, A2, 1 L DNAJ gene family), as well as, genes responsible for oxidative protein folding in the ER such as ERO1α, ERP44, and PDI. The latter genes upregulation contributes to the cells' ability to cope with ER stress. However, when the stress is prolonged the oxidative activity of ERO1α and other oxireductases burden cells with potentially toxic reactive oxygen species,[10] which may contribute to healing impairment. Chronic UPR activation may also lead to apoptotic death through CHOP (red box), a master regulator of ER stress induced apoptosis that inhibits pro-survival BCL2 and GADD34, which exacerbates cell death signalling. IRE1α can also contribute to cell death by activating the JNK pathway through a direct interaction with TRAF2 and ASK1 (red boxes) followed by induction of the pro-apoptotic factor BAX (which can also bind to IRE1 α and release $Ca^{2+}$ from the ER). 4-PBA, a small molecular chemical chaperone that hampers protein misfolding and aggregation, as well as, promotes intracellular trafficking and secretion,[15] reduces ER stress and may thus improve healing of chronic wounds

marker upregulation. Thus, UPR activation appears to be part of normal healthy skin wound healing in the elderly, however, because of the vast literature on the pathological role of ER stress in various chronic diseases[4] we rationalise that it may play a detrimental role in chronic leg ulcers such as venous ulcers. Indeed, ER stress was associated with downstream apoptotic markers such as *ASK1*, *TRAF2 BCL2*, *BAX* and *BAG* (Figure 1E).

## 3.2 | ER stress in aged skin

During ageing, the function of the UPR is compromised, as there is a progressive failure of the chaperoning systems and a decline in UPR components so that UPR activation cannot rescue the ER stress.[26] This has been shown in several tissue types, however, not in skin. When establishing our in vitro ER stress wound healing model, we found that keratinocytes from older leg

donors showed higher expression of UPR markers in unstimulated conditions compared with young truncal skin keratinocytes (Figure 5). Thus, it may be that these cells are primed for impaired healing. As the phenotype persists ex vivo in the cells, it suggests that stable cellular alterations such as senescence or epigenetic changes are at play. It would certainly be worthwhile testing if chemical chaperones or other drugs targeting the UPR would impact other skin aging phenotypes in addition to impaired wound healing; for example, skin atrophy.

### 3.3 | Impact of ER stress on cell migration and proliferation

Cell migration is an essential basic part of wound healing and reepithelialisation in fact defines when a wound is healed. While many studies have examined cell migration in other contexts, none has studied how ER stress impacts keratinocyte and dermal fibroblast migration. Firstly, we established that known ER stress inducing drugs impaired cell migration and that this was partly reversed by the chemical chaperones 4-PBA and TUDCA (Figure 2). Secondly, cell cultures from aged and diseased leg donors showed a high basal ER stress and low migratory capacity when compared with young control cells that did not exhibit any basal ER stress. Thirdly, we found that the chemical chaperones dramatically improved the migratory capacity of the elderly leg keratinocytes, but not of the dermal fibroblasts. Fourthly, we found ER dilation, indicative of ER stress, in wound edge keratinocytes. Our interpretation of these findings is that ER stress is a viable target mainly in diseased keratinocytes. While 4-PBA has not been used previously on wounds, it has been used on keratinocyte cultures and skin equivalents derived from healthy donors and epidermolysis bullosa simplex patients.[27] In this ambitious study, it had various positive as well as negative effects, among the latter was impaired migration and induction of epidermal splits similar to epidermolysis bullosa simplex pathology; however, high 4-PBA concentrations in the millimolar range was used while we used low micromolar levels. ER proteostasis is a fine balance and different chaperone concentrations are likely to yield varying results including toxic off target effects that are probable at high concentrations.

Important for healing besides migration is cellular proliferation. Whether ER stress induces or inhibits proliferation is a complex question, and very much organ and context dependent. The chronic wound environment it itself contains several factors such as inflammatory mediators that are capable of inhibiting proliferation.[28] In our in vitro scratch assay experiments on keratinocytes that were performed with and without the proliferation inhibitor mitomycin C, it appeared that ER stress primarily reduces cell migration, however, with a minor yet significant impairment also of proliferation (Figure 3). In cancer, which biology has many similarities to chronic wounds, it was shown the ER stress and the UPR are involved in tumour-stimulated angiogenesis[18,29] and tumour expansion.[30,31] In pancreatic islets, it was shown that PERK-deficient mice exhibit impaired beta cell proliferation, which results in neonatal diabetes.[32] However, in another study alleviating ER stress by reducing insulin production in mice actually promotes beta cell proliferation.[33]

### 3.4 | Pharmacological treatment of ER stress

There is a growing list of inhibitors and activators of the UPR[34]; however, none of these has reached routine clinical use. 4-PBA, approved for the treatment of urea cycle disorders, and TUDCA, approved for primary biliary cholangitis, are chemical chaperones that are assumed to help protein folding and thereby reducing the ER stress, although their precise action is not understood.[35,36] One theoretical advantage of 4-PBA is that it acts upstream in the UPR as compared with many novel targeted experimental drugs.

There are many ongoing clinical trials on the systemic use of these agents in several diseases including diabetes and obesity.[37] It is important to stress that elderly patients suffering from chronic leg ulcers are not an attractive target group for the pharmaceutical industry as any successful treatment would only be given for a limited time combined with the fact that life expectancy is relatively short in these patients. Thus, drug repurposing is much more realistic than costly novel drug development.

Although many studies use various animal models to study wound healing, human wound healing differs substantially[17] and no animal model exists for venous ulcers. To best examine how ER stress relief impacts wound healing and particularly reepithelialisation, because of our keratinocyte migration results (Figures 2 and 3), we opted for the organotypic ex vivo human skin model.[14] As the ex vivo skin flaps are ischaemic they are quite likely to have profound ER stress as it is well established that ischemia/hypoxia triggers ER stress,[38,39] and they are also relevant to venous ulcers that are hypoxic in nature.[40] Systemic 4-PBA was indeed reported to protect rat skin flaps against ischaemia-reperfusion injury and apoptosis by inhibiting ER stress.[41] As 4-PBA had the greatest impact on cell migration (Figure 2), we examined this compound's effect and found that it

substantially speeded up wound healing (Figure 6). While 4-PBA may have some adverse effects when systemically used in humans, for example, amenorrhea and neurotoxicity,[42] these are most likely avoided with topical application. Indeed, 4-PBA was successfully used topically in mouse models of glaucoma[43] and acute irritant dermatitis.[44] 4-PBA was even found to reverse the detrimental topical effects of the chemical warfare agent lewisite, including ER stress.[45] Considering the growing need for improved treatments, we suggest that the next logical step would be clinical trials in which the compound is applied topically to venous ulcers.

## 4 | METHODS

### 4.1 | Human wound biopsies

Healthy volunteers (>60 years of age) were enrolled at Karolinska University Hospital Dermatology Clinic. Full-thickness skin wounds were made with a 4-mm biopsy punch in two adjacent spots 2 cm apart on the distal lower leg of the healthy volunteers—the excised skin was then used as controls for the following wounds. On day 1 and day 7 after injury, the wound edge area was excised with a 6-mm biopsy punch from one of the existing wounds (n = 11). Similar 4 mm wound edge biopsies (n = 7) were obtained at one occasion from patients (>60 years of age) with chronic C6 venous ulcers for >3 months that were defined according to the 2004 CEAP classification.[46] All samples were snap frozen in liquid nitrogen. The clinical material was obtained after oral and written informed patient consent, and the study was approved by the Stockholm Regional Ethics Committee and conducted in accordance with the principles of the Declaration of Helsinki.

### 4.2 | nCounter gene expression assay

Human biopsies from healthy volunteers and patients with venous leg ulcers were collected, as described above, and total RNA was extracted and kept at −80°C. We applied the nCounter in-solution hybridization method using nCounter Sprint platform (NanoString Technologies, Inc. Seattle, WA) to measure the gene expression levels of candidate genes, as previously described.[47]

### 4.3 | Electron microscopy

Skin wound biopsies were fixed in glutaraldehyde, embedded in epoxy resin and selected areas were sectioned for transmission electron microscopy observation using the FEI Technai G2 instrument.

### 4.4 | Human ex vivo wound model

Normal full-thickness human skin samples were obtained from abdominal reduction surgeries of healthy young donors (Nordiska Kliniken, Stockholm) and used for ex vivo wound healing as described.[14] For topical treatment, 4-PBA or vehicle ($H_2O$) were dissolved in 30% pluronic F-127 gel (Sigma-Aldrich) and applied on the wounds immediately following injury and then every other day. For transdermal treatment-4-PBA and vehicle were dissolved in media. Wound samples were collected for histological analysis at the indicated time points.

### 4.5 | Statistical analysis

Statistical significance was determined by the two tailed Student's *t* test or two-way analysis of variance with Dunnett's multiple comparison test using GraphPad Prism Version 7. *P* value <.05 was considered significant. Further Method details are given in the Supporting Information.


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## AUTHOR CONTRIBUTIONS

**Etty Bachar-Wikstrom** and **Jakob D. Wikstrom:** Designed the study. **Etty Bachar-Wikstrom**, **Mansi Manchanda**, **Ritu Bansal**, **Magnus Karlsson**, **Paula Kelly-Pettersson**, and **Jakob D. Wikstrom:** Performed experiments. **Etty Bachar-Wikstrom**, **Mansi Manchanda**, **Ritu Bansal**, and **Jakob D. Wikstrom:** Performed analysis. All authors contributed to writing.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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