Reduced Iron in Diabetic Wounds: An Oxidative Stress-Dependent Role for STEAP3 in Extracellular Matrix Deposition and Remodeling

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Iron is crucial for maintaining normal bodily function with well-documented roles in erythropoiesis, hemostasis, and inflammation. Despite this, little is known about the temporal regulation of iron during wound healing, or how iron contributes to wound biology and pathology. In this study, we profiled tissue iron levels across a healing time-course, identifying iron accumulation during late-stage repair. Diabetic murine wounds displayed significantly reduced iron levels, delayed extracellular matrix deposition, and dysregulation of iron gene expression. In vitro studies revealed important cellular roles for iron, promoting both the deposition and remodeling of extracellular proteins. Functional studies identified oxidative stress-dependent upregulation of the iron-converting metalloreductase, STEAP3, as a key mediator of extracellular matrix production. Taken together, these data reveal a mechanistic role for iron in facilitating the remodeling stage of wound healing. Indeed, targeting tissue iron could be a promising future strategy to tackle the development and progression of chronic wounds.

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INTRODUCTION

Iron is one of the body’s most abundant trace elements, responsible for orchestrating the action and inaction of a diverse range of cellular functions. The importance of iron pertains to its ability to alter its oxidative state, in which it drives biological processes that require electron transfer, such as oxidative phosphorylation and DNA synthesis and repair (Bogdan et al., 2016). However, in its biologically active state (Fe²⁺), iron reduces oxygen to form free radicals that cause cellular damage (Ray et al., 2012). Thus, crucial mechanisms have evolved to tightly regulate bodily iron fluxes and prevent toxicity.

Iron (Fe³⁺) is exported into the bloodstream, where it binds mainly to transferrin (Tf). Tf-Fe then binds to Tf receptor 1 at the cell surface (Arezes and Nemeth, 2015), the Tf-Tf receptor 1–complex is internalized through clathrin-mediated endocytosis, and Fe³⁺ is released (Mayle et al., 2012). Fe³⁺ is reduced to Fe²⁺ by the ferrireductase, STEAP3 (six-transmembrane epithelial antigen of prostate), with Fe²⁺ transported into the cytoplasm by divalent metal transporter 1 (Muckenthaler et al., 2017). Intracellular iron can then be stored (in ferritin) or exported via the only known cellular iron exporter, ferroportin (Ward and Kaplan, 2012).

Despite this sophisticated regulation, there exist a number of iron deficiency or iron overload disorders (Arezes and Nemeth, 2015) that are linked to chronic pathologies (e.g., cardiovascular disease [Qi et al., 2007] and fatty liver disease [Valenti et al., 2012]). Indeed, iron loading causes insulin resistance and increases glucose uptake in mice (Huang et al., 2013), and elevated iron is associated with increased risk of type 2 diabetes mellitus in humans (Eshak et al., 2018). The effects of iron in mediating diabetes risk are further shown in the iron loading disorder hereditary hemochromatosis, which increases apoptosis of pancreatic β cells (Cooksey et al., 2004), contributes to abnormal glucose homeostasis, and increases the prevalence of diabetes in those affected (Barton and Acton, 2017; McClain et al., 2006). Thus, altered iron metabolism mediates many of the risks factors for the development of type 2 diabetes mellitus.

In wound healing, iron is known for its oxidative role in hemostasis, in which ferrous iron is released from hemoglobin (Lipinski and Pretorius, 2012) and acts to promote blood clotting (Kell and Pretorius, 2015; Pretorius et al., 2013). Abundant wound heme also directs inflammation and the release of hydroxyl radicals (Yeoh-Ellerton and Stacey, 2003), influences monocyte differentiation into macrophages (Haldar et al., 2014), and Fe²⁺ itself retains effects on macrophage function and polarization (Agoro et al., 2018; Kroner et al., 2014; Sindrilaru et al., 2011; Sindrilaru and Scharfetter-Kochanek, 2013). Iron-dependent enzymes are also required for stimulating angiogenesis (Loenarz and Schofield, 2008; Ozer and Bruick, 2007) and are involved
in the post-translational stabilization of collagen (Hutton et al., 1967; Markolovic et al., 2015). Despite this, little is known about the spatial or temporal distribution of endogenous iron in cutaneous wound biology, or how it links to pathological healing.

In this study, we report temporal changes in endogenous iron distribution during normal acute wound healing and across a pathological murine model. We determine a specific, and to our knowledge previously unreported, role for iron in aiding extracellular matrix (ECM) remodeling and deposition. Finally, we explore the mechanistic importance of iron-induced oxidative stress via STEAP3, thus elucidating a fundamental role for iron in ECM distribution during cutaneous wound repair.

RESULTS
Iron accumulates during the remodeling phase of wound healing
Inductively coupled plasma-mass spectrometry (ICP-MS), the gold standard technique for measuring metal abundance (Lee et al., 2014), was used to characterize tissue iron concentrations across a normal healing time-course. Wounds were evaluated at day 1, 3, 7, and 14 postwounding (PW) in C57/Bl6 mice, and iron abundance was compared with unwounded skin (D0) (Figure 1a). A substantial increase in tissue iron concentration was observed at day 7 and day 14 PW, compared with D0 (P < 0.001). Perls’ Prussian blue histological staining independently confirmed the ICP-MS data (Figure 1b), in which quantification (Figure 1c) showed an increased iron deposition at day 7 (P < 0.001) and day 14 PW (P < 0.001). The deposition of iron at day 7 correlated temporally with wound collagen production (Figure 1d–f, Supplementary Figure S1a) and increased Col3a1 (P < 0.01) and Col1a1 (P < 0.001) mRNA (Supplementary Figure S1d).

Diabetic wound pathology is characterized by impaired iron accretion and reduced ECM deposition
We then asked whether the delayed healing of diabetic (Db) wounds (Wilkinson et al., 2019) altered local iron levels. Specifically, ICP-MS /ICP-optical emission spectrometry was used to evaluate iron abundance in nondiabetic (NDb) and Db skin (D0) and wounds at day 3 and day 7 PW (Figure 2a). As in wild-type mice (Figure 1a), NDb wounds displayed significantly elevated iron at day 7 versus D0 (P < 0.01). At all time points, Db skin (P < 0.001) and wounds (day 3, P < 0.01; day 7, P < 0.001) presented with significantly reduced iron compared with NDb. Once again Perls’ Prussian blue histology confirmed metal profiling data, showing reduced iron deposition in the Db model at day 3, 7, and 14 (P < 0.001; Figure 2b and c).

In line with the observed iron changes, Db wounds displayed reduced collagen deposition at day 7 PW (Figure 2d–g, Supplementary Figure S1b and c) and impaired Col3a1 and Col1a1 transcription (Supplementary Figure S1e). Indeed, immature and mature ECM fiber analyses (picrosirius red) confirmed reduced mature fibers in Db wounds (Supplementary Figure S2). Collectively, these data highlight a strong correlation between iron levels in wound tissue and deposition of wound ECM.

Iron administration increases intracellular iron storage
In order to evaluate the direct effects of iron on ECM production and deposition, we investigated an in vitro model. Human dermal fibroblasts (HDFs) were treated with ferric ammonium citrate (FAC) for up to 11 days. The highest concentration of FAC tested (100 μM) increased cell growth (Figure 3a) and was nontoxic (Figure 3b). A ferrozine assay (Riemer et al., 2004) directly quantified the increased intracellular iron in 100 μM FAC-treated HDFs (Figure 3c). Moreover, treatment with 100 μM FAC resulted in high cytoplasmic ferritin stores, demonstrated by western blot (day 5, P < 0.05; Figure 3d) and immunocytochemistry (P < 0.001; Figure 3e and f). Thus, HDFs internalized and stored iron from exogenously administered FAC.

FAC treatment of HDFs dose-dependently increases fibronectin remodeling.
Treatment of 100 μM FAC led to a reduced endogenous fibronectin deposition compared with 10 μM FAC (P < 0.01; Figure 4a and b). Next, we performed an Fn-488 assay (Cytoskeleton, Denver, CO) to assess the influence of FAC on the remodeling of exogenous fibronectin. HDFs treated with 100 μM FAC for 24 hours demonstrated substantially increased remodeling (P < 0.01; increased 488 intensity), whereas treatment with ARP101, an inhibitor of matrix metalloproteinase (MMP) 2, significantly reduced remodeling in the presence of FAC (P < 0.01; Figure 4c and d). As MMP2 inhibition reduced fibronectin remodeling, MMP2 activity in HDFs was assessed by gelatin zymography. Here, FAC treatment significantly increased activity of the protease MMP2 (P < 0.05; Figure 4f and g). Confirmation that MMP2 degrades fibronectin is provided in Supplementary Figure S3a. Zymography performed on MMP2 and MMP9 standards incubated with FAC excluded direct inhibition of MMP activity (Supplementary Figure S3b–d). In HDF cells, increased MMP2 expression was observed following 100 μM FAC treatment (P < 0.01; Figure 4f). Clearly, high levels of FAC increased MMP2 activity in HDFs, with MMP2 being able to remodel fibronectin in vitro.

FAC administration accelerates extracellular deposition of type I and type III collagen
A hydroxyproline assay was used to assess the collagen content in HDFs treated with FAC. Significantly increased hydroxyproline was observed after treatment with 100 μM FAC (P < 0.001; Figure 4g). This was confirmed by immunochemistry, in which 100 μM FAC significantly increased the deposition of collagen III (P < 0.01) and collagen I (P < 0.001) in HDFs in vitro at day 11 (Figure 4h and i). These assays were also performed on a range of FAC treatments, in which we saw a dose-dependent increase in collagen. Cotreatment with the iron chelator, deferoxamine (100 μM), attenuated this increase in collagen, whereas the non-iron oxidant phorbol 12-myristate 13-acetate (200 nM) failed to increase collagen levels, demonstrating an iron-specific effect (Supplementary Figure S3e and f). Finally, scanning electron microscopy allowed for the determination of structural changes after FAC treatment at a higher resolution than confocal microscopy. Intriguingly, 100 μM FAC treatment led to large microvesicles and increased extracellular secretion versus the control group (Figure 4j). Taken...
together, these data indicate that iron administration leads to significant ECM deposition.

Cytoplasmic iron loading leads to oxidative stress-induced ECM production via a STEAP3-dependent mechanism

As iron is known to cause free radical production (reviewed in Bresgen and Eckl, 2015), we evaluated oxidative stress in FAC-treated HDFs. A CellROX assay (Thermo Fisher Scientific, Waltham, MA) showed that FAC treatment increased oxidative stress in HDFs, which was attenuated by deferoxamine ($P < 0.05$; Supplementary Figure S4a and b) and the antioxidant, mannitol ($P < 0.05$; Figure 5a and b). Mannitol treatment also reduced the FAC-mediated secretion of collagen III ($P < 0.001$; Figure 5a and c) and collagen I ($P < 0.01$; Figure 5a and d). To explore the mechanistic link between FAC-induced oxidative stress and subsequent ECM deposition, a tissue expression screen of selected iron-associated genes was performed (Supplementary Figure S4c). In this study, Steap3 was elevated in day 7 wild-type wounds ($P < 0.05$; Figure 5e) and decreased in day 7 Db wounds ($P < 0.01$; Figure 5f). STEAP3 was significantly increased in HDFs after 100 µM FAC administration at day 3 ($P < 0.05$; Figure 5g). Thus, both oxidative stress and STEAP3 appear crucial for FAC-mediated ECM deposition.
Finally, we assessed the functional link between oxidative stress, STEAP3, and ECM production. Treatment of FAC-loaded HDFs with the antioxidant mannitol attenuated STEAP3 expression ($P < 0.05$; Figure 5h) and reduced HDF ferritin stores ($P < 0.001$; Supplementary Figure S4d). Next, small interfering RNA (siRNA) was used to target STEAP3 in HDFs, and its role in iron-induced collagen production was assessed. Quantitative real-time reverse transcriptase–PCR demonstrated significant, stable knockdown of STEAP3 (Supplementary Figure S4e). In HDFs treated with STEAP3 siRNA, extracellular production of collagen II (Figure 5i and j) and collagen I (Supplementary Figure S4f and g) was significantly reduced after treating with 100 µM FAC (compared with control siRNA). Finally, siRNA targeting STEAP3 led to reduced oxidative stress in the presence of 100 µM FAC ($P < 0.001$; Figure 5k). Taken together, these data suggest that FAC-induced oxidative stress mediates collagen deposition, partly through STEAP3.

Figure 2. Diabetic wounds display impaired iron accumulation and delayed collagen deposition. (a) Db skin and wounds possessed significantly less iron than NDb (n = 5 per group). The # symbol represents NDb versus Db. (b) PPB staining where arrows represent deposits and spleen represents the PPB positive control. DAPI is used as counterstain and (c) iron abundance quantification. (d) Immunocytochemistry staining for (e) fibronectin, (f) collagen III and (g) collagen I. Mean ± SEM. *$P < 0.05$, **$P < 0.001$, red *, # $P < 0.001$. D0, normal skin; D3, day 3 post wounding; D7, day 7 post wounding; D14, day 14 post wounding; Db, diabetic; NDb, nondiabetic; PPB, Perls’ Prussian blue; SEM, standard error of the mean. Bar = 50 µm.
DISCUSSION
Iron is a vital trace element that is essential for life. Its role as a redox active metal is well-known, as is its major function in erythropoiesis (Majmundar et al., 2010). In this study, and to our knowledge previously unreported, we provide findings that show the temporal regulation of iron during murine wound healing and reveal its impairment in Db wound pathology. To date, iron has been largely recognized for driving control growth. 

**Figure 3. Human dermal fibroblasts sequester administered iron.** (a) HDFs growth kinematics and (b) viability after FAC treatment. Sequestration of FAC determined by (c) ferrozine, (d) western blot, and (e) immunocytochemistry and (f) quantified. Ferritin statistics versus 100 μm FAC. Ferritin = red. DAPI = counterstain. Arrows = ferritin. n = 3 donors. Mean ± SEM. *P < 0.05, **P < 0.001, red *P < 0.001. CTCF, corrected total cell fluorescence; D2, day 2 post wounding; D5, day 5 post wounding; D11, day 11 post wounding; DEF, deferoxamine iron chelator plus 100 μm FAC; EtOH, ethanol control; FAC, ferric ammonium citrate; HDF, human dermal fibroblasts; PI, propidium iodide. SEM, standard error of the mean. Bar = 50 μm.
hemostasis (Lipinski and Pretorius, 2012) and modulating inflammatory cell behavior (Agoro et al., 2018; Kroner et al., 2014) during the early-stage wound healing processes. Our data demonstrates that iron plays a major role in modulating dermal ECM deposition and remodeling that characterizes late-stage wound repair (Young and McNaught, 2011). Previous groups have measured iron in experimental rat wounds using flame atomic absorption spectroscopy (Lansdown et al., 2015). Figure 4. Iron administration causes fibronectin remodeling and accelerated collagen deposition in vitro. (a) HDFs treated with FAC (11 days) and (b) stained for fibronectin. (c) Fn-488 remodeling. (d) Images of ARP101. (e) Zymography of MMP2 inhibitor groups plus 100 μM FAC. A = control; B = 10 μM FAC; C = 100 μM FAC. (f) Hydroxyproline and COL III and I. (g) MMP2 qRT-PCR. (h) Immunocytochemistry and (i) quantification. Collagens = green. Bar = 50 μm. (j) Scanning electron microscopy. Secretions = gray arrows. Microvesicles = white arrows. Bar = 10 μm and Inset bar = 2.5 μm. n = 3 donors. Mean ± SEM. *P < 0.05, **P < 0.01, red *P < 0.001. COL, collagen type; FAC, ferric ammonium citrate; HDF, human dermal fibroblasts; MMP, matrix metalloproteinase; SEM, standard error of the mean.
1999) and in chronic wound exudate using ICP-optical emission spectroscopy (Yeoh-Ellerton and Stacey, 2003). However, to our knowledge, there are no previous reports comparing endogenous iron between acute and pathological murine wound tissue using the more sensitive and accurate technique of ICP-MS.
Previous in vitro studies have shown that HDFs readily uptake and store administered iron, which aids cellular proliferation (Jenkins et al., 2011; Le and Richardson, 2002). In this study, we show that iron stimulates HDFs to increase MMP2 expression, which could contribute to remodeling of fibronectin in vitro. Indeed, MMP2 has previously been shown to successfully cleave fibronectin (Jiao et al., 2012), an ECM component required during hemostasis and granulation tissue formation for epithelial migration and cellular adhesion (Barker and Engler, 2017; Leiss et al., 2008).

Despite its crucial roles in early repair, stronger and more stable collagen fibers replace fibronectin during dermal remodeling (Lenselink, 2015). Our data demonstrate that iron strongly stimulates extracellular deposition of both collagen type III and collagen type I in vitro. In hereditary hemochromatosis, patients show local inflammation and fibrosis (Wang et al., 2017; reviewed by Wood et al., 2008), although this is most likely an indirect result of comorbidities known to contribute to fibrosis (e.g., alcohol consumption; Bataller et al., 2003). This fits with the observation that in experimental hemochromatosis mouse models, iron loading occurs in the liver but is not correlated with increased ECM production (Subramaniam et al., 2012).

Experiments were performed exclusively in female mice. However, the widely reported sex differences in healing (Gilliver et al., 2008), future studies could assess sex variation in the role of iron in wound repair. Similarly, although the db/db mouse model is widely used, it fails to fully replicate human chronic wounds. A future priority is to confirm the role of iron in human Db wound pathology.

Iron delivery in vitro aids spheroid growth and ECM production in rat aortic smooth muscle cells (Casco et al., 2017), causes fibrogenesis in murine hepatic stellate cells (Mehta et al., 2018), and increases proliferation in human myeloid progenitor cells (Pourecelot et al., 2015). As a potential mechanism, iron loading is known to induce cellular oxidative stress through the release of hydroxyl radicals (Gao et al., 2009). In turn, hydrogen peroxide-induced oxidative stress promotes collagen production in rat cardiac fibroblasts via NAD(P)H oxidase (Nox; Wang et al., 2013), and induces fibrosis in human hepatic stellate cells in vitro (Andueza et al., 2018). More recently, high levels of protein oxidation have been linked to idiopathic pulmonary fibrosis and bleomycin and transforming growth factor-β–induced murine fibrosis models (Anathy et al., 2018). Finally, systemic sclerosis skin fibroblasts (from patients and mice) that possess excessive dermal ECM are characterized by heightened reactive oxygen species (Kavian et al., 2010; Sambo et al., 2001), which can be downregulated by the antioxidant stimulator, nuclear factor-like 2 (Kavian et al., 2018). Thus, we and others have showed that oxidative stress is a potential contributor to accelerated ECM deposition in a variety of contexts.

A tissue screen of iron-related genes elucidated upregulation of Steap3 at day 7 PW, yet Db wounds displayed an altered profile of iron gene expression with significant downregulation of Steap3 compared with NDb wounds. In fact, targeting of STEAP3, which is required for iron transport into the cytoplasm via divalent metal transporter 1 (Ohgami et al., 2005), led to a reduced collagen deposition in vitro. In silico, Han et al. (2018) recently demonstrated that STEAP3-associated genes are linked to several cellular functions, including ECM organization. Finally, antioxidant treatment in vitro dampened STEAP3 expression in iron-stimulated HDFs, and siRNA targeting of STEAP3 reduced oxidative stress, thus implying a direct role for STEAP3 in oxidative stress-induced ECM deposition.

Taken together, these data demonstrate a previously unappreciated role for iron in late-stage wound repair in vivo, with clearly reduced levels in murine pathological healing. Of therapeutic interest, endosomal conversion of exogenously administered iron via STEAP3 could accelerate ECM deposition through an oxidative stress-dependent mechanism. Further studies, particularly using human tissue, are essential to explore the feasibility of directly manipulating tissue iron levels to promote wound healing.

**MATERIALS AND METHODS**

**Animal experimentation**

Mice, purchased from Envigo (Cambridgeshire, United Kingdom), were housed at the Biological Services Facility at the University of Manchester (Manchester, United Kingdom) with ad libitum access to food and water. All animal experimentation was performed according to UK Home Office regulations under project licence 70/8136.

**Wounding experiments**

Female wild-type C57Bl6, NDb (Lepr<sup>+/−</sup>) and Db (Lepr<sup>−/−</sup>) mice were anesthetized and wounded at 8–10-weeks-old using our established protocol (Ashcroft and Mills, 2002) with modifications. Two equidistant 6-mm-excisional wounds were created with trace metal free titanium instruments (World Precision Instruments, Hertfordshire, United Kingdom) on the dorsum of each mouse. Mice were administered buprenorphine postoperatively. For the wild-type time-course experiments, wounds were collected at day 1, 3, 7, and 14 PW. For NDb versus Db time-course experiments, wounds were collected at day 3 and day 7 PW only. Normal skin (D0) was also collected. Further details are provided in the Supplementary Materials.

**Metal quantification**

The samples were analyzed by a combination of ICP-MS, one of the most sensitive, commercially available techniques for element analysis (Liu et al., 2014) and ICP-optical emission spectrometry. Tissue frozen at −80 °C was freeze-dried in trace metal free vacuum containers (BD Biosciences, Wokingham, United Kingdom) at −50 °C and 0.03–0.04 Mbar. Freeze-dried samples, along with certified reference material (DOLT-5 dogfish liver; National Research Council Canada, Ottawa, Canada) were prepared in trace metal free nitric acid (Sigma-Aldrich, Dorset, United Kingdom) and 30% hydrogen peroxide (Sigma-Aldrich) as in Ouypornkochagorn and Feldmann (2010) and digested as described in the Supplementary Materials. Samples were subsequently analyzed on an Agilent 7500cx ICP-MS (Agilent Technologies, Cheshire, United Kingdom) or Optima 5300DV ICP-optical emission spectrometer (PerkinElmer, Buckinghamshire, United Kingdom).

**Histological analysis**

Tissue samples were stained with Perls’ Prussian blue to detect iron deposits. Immunohistochemistry was performed using mouse anti-fibronectin (clone EPS; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-collagen III (ab7778) and rabbit anti-collagen I.
(clone EPR7785; Abcam, Cambridge, United Kingdom) primary antibodies. Further details are provided in the Supplementary Materials.

Quantitative real-time reverse transcriptase–PCR
RNA was extracted from wounds, normal skin tissue and cells. Full information for quantitative real-time reverse transcriptase–PCR experiments are in the Supplementary Materials with primer sequences provided in Supplementary Table S1.

Fibroblast culture
Primary HDFs were isolated from human skin from donors aged <50 years, obtained with institutional approval and full, written informed consent from Castle Hill Hospital, Hull, United Kingdom (17/SC/0220) as previously described (Wilkinson et al., 2019). HDFs were cultured in MEM (Thermo Fisher Scientific) containing 10% fetal bovine serum and 1% penicillin-streptomycin solution (Thermo Fisher Scientific). FAC was used as described in the Supplementary Materials. Growth and viability with FAC treatment were determined. ECM production was assessed by immunocytochemistry, western blotting, and scanning electron microscopy, as described in the Supplementary Materials.

siRNA experiments
HDFs were transfected with two different validated Silencer Select siRNAs (Thermo Fisher Scientific) targeting STEAP3 mRNA. Transfection was achieved using Lipofectamine RNAiMAX in Opti-MEM (Thermo Fisher Scientific) following the manufacturer’s instructions. After 6 hours, media was replaced with fresh antibiotic-free MEM containing 2% fetal bovine serum, and HDFs were treated with FAC for 5 days and analyzed (quantitative real-time reverse transcriptase–PCR, immunocytochemistry, and oxidative stress). Silencer Select Negative Control No. 1 (Thermo Fisher Scientific) siRNA was used as a validated control.

Oxidative stress experiments
Oxidative stress was measured in HDFs after FAC treatment using CellROX Green Reagent (Thermo Fisher Scientific). HDFs were imaged using confocal microscopy (LSM 710; Carl Zeiss, Cambridge, United Kingdom) at ×20 magnification and intensity of staining (corrected total cell fluorescence) was determined (McCoy et al., 2014). To inhibit oxidative stress, 1 mM mannitol (Thermo Fisher Scientific) was added at the time of FAC administration.

Statistical analysis
Mean ± standard error of the mean were used for all data sets. Statistical analyses were performed on all quantitative data using GraphPad Prism 7 (GraphPad Software; San Diego, CA). Independent t tests, one-way analysis of variance and two-way analysis of variance were used, with post hoc analyses (Dunnett and Tukey tests, one-way analysis of variance and two-way analysis of variance) required for transferrin-induced hepcidin expression. 41:274–86.

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AUTHOR CONTRIBUTIONS
Conceptualization: HNW, MJH; Formal Analysis: HNW; Funding Acquisition: MJH; Investigation: HNW, SEU, KLB, RK; Methodology: HNW, MJH; Project Administration: HNW, MJH; Resources: KAM, MJH; Supervision: MJH; Validation: HNW, SEU, KLB, RK; Visualization: HNW; Writing - Original Draft Preparation: HNW; Writing - Review and Editing: HNW, KAM, MJH.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.05.014.

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Wound collection
Normal skin and wounds were placed in neutral buffered formalin for wax histology or flash frozen in liquid nitrogen and stored at −80 °C for biochemical analysis and metal quantification.

Metal quantification
Freeze-dried skin and wound samples, prepared in a 50:50 mixture of concentrated nitric acid and hydrogen peroxide, were digested in a MARS 6 microwave with a MARSXPRESS vessel (CEM Microwave Technology, Buckingham, United Kingdom) at 400W power. The following cycling parameters were used: ramp to 75 °C for 5 minutes, hold at 75 °C for 30 minutes, ramp to 95 °C for 5 minutes, and a final hold at 95 °C for 60 minutes. Once cooled, ultrapure trace metal free water (18.2 Ω) was added to each sample to give 25% final nitric acid concentration. Internal standards ranging from 1–100 parts per billion were prepared in 25% nitric acid and used as calibration. A water reference standard (CRM 1643E) was also included for verification of the internal standards.

Histological analysis
Sections, cut at 5 μm thickness, were dewaxed (xylene) and brought to water before staining. Perls’ Prussian blue staining was performed to detect iron deposits in skin and wound tissue. A 5% potassium ferrocyanide:5% hydrochloric acid mixture was flooded over sections for 10 minutes. Sections were counterstained (neutral red), mounted in PERTEX mounting medium (Cell Path, Shrewsbury, United Kingdom) and imaged using a brightfield microscope (Eclipse E400; Nikon, Tokyo, Japan) with SPOT camera (Image Solutions, MI). Picrosirius red staining was used to analyze matrix fiber composition as in Wilkinson et al. (2018). Immunohistochemistry was performed using described antibodies (main text) with appropriate Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific). Slides were mounted in Mowiol-488 (Sigma-Aldrich) with DAPI (Thermo Fisher Scientific) and imaged on an LSM 710 confocal microscope (Carl Zeiss) at ×20 magnification. DAPI (nuclei), Alexa Fluor 488 (fibronectin) and Alexa Fluor 594 (collagen III and collagen I) were excited using the 405 nm diode, 488 nm argon, and 561 nm diode pumped solid state lasers, respectively. Intensity staining analysis was performed in ImageJ software, version 1.8.0 (National Institutes of Health, Bethesda, MD).

Quantitative real-time PCR
RNA was extracted from wounds, normal skin tissue and cells. Tissue was homogenised (T10 ULTRA-TURRAX; IKA, Oxford, United Kingdom) in Invitrogen Ambion TRIzol reagent (Thermo Fisher Scientific) and phase separation performed with chloroform. RNA was purified using an Invitrogen Ambion PureLink RNA Mini Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. RNA was adjusted to 1 μg/10 μl, determined by a NanoDrop UV-Vis spectrophotometer (ND-1000; Thermo Fisher Scientific), and reverse transcribed with random primers (Promega, Madison, WI) and Bioresearch (Bioline, London, United Kingdom). cDNA was amplified on a CFX Connect thermocycler (Bio-Rad, Hertfordshire, United Kingdom) with 2X Takyon SYBR Mastermix (Eurogentec, Hampshire, United Kingdom). Primer sequences for quantitative real-time reverse transcriptase—PCR experiments are provided in the supporting information (see Supplementary Table S1).

Cell growth and viability
HDFs were grown in the presence of FAC for 11 days and cell counted for growth kinetics determination. For viability assessment, HDFs were stained with 1% propidium iodide (Thermo Fisher Scientific) and analyzed on a FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences, Oxford, United Kingdom).

Ferrozine assay
Cells were scraped on ice in 50 mM (w/v) sodium hydroxide, containing 1% HALT protease inhibitor cocktail (Thermo Fisher Scientific). Lysates (100 μl) were then used for a ferrozine assay to measure intracellular iron content as in Riemer et al. (2004). Cellular iron concentration (μM) was determined from a FAC standard curve.

Immunocytochemistry
HDFs were cultured for 11 days on glass coverslips, fixed in 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and stained for mouse anti-COL1A1 (clone 3G3), mouse anti-COL3A1 (clone B10), mouse anti-fibronectin (clone EP5; Santa Cruz Biotechnology), and rat anti-ferritin (clone EPR3004Y; Abcam). Alexa Fluor 488- and 594-conjugated secondary antibodies (Thermo Fisher Scientific) were used. Coverslips were mounted, and images were taken by confocal microscopy, as previously described.

Western blot
Following 5 days of FAC treatment, HDFs were lysed with radioimmunoprecipitation assay buffer and loaded on 10% SDS-PAGE gels. A Precision Plus Protein Kaleidoscope Pre-stained Protein ladder (Bio-Rad Laboratories) was used. Proteins were transferred onto 0.2 μm nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom), blocked in 5% (w/v) skimmed milk and probed for mouse anti-ferritin (clone B12; Santa Cruz Biotechnology), Mouse anti-β-actin (clone AC-15; Sigma-Aldrich) as a loading control. HRP-linked secondary antibody (GE Healthcare) and chemiluminescent substrate (Pierce ECL Plus; Thermo Fisher Scientific) was used for detection. Films were developed and quantified in ImageJ software, version1.8.0.

Hydroxyproline assay
HDFs were collected after 11 days of treatment and subjected to hydroxyproline determination using a hydroxyproline assay kit (Abcam) according to the manufacturer’s guidelines.

Fn-488 remodeling
HDFs were seeded into 12-well μ-slides (Ibidi, Munich, Germany) at 2.5 × 10^5 cells/ml media. Media was replaced with MEM containing 2% fetal bovine serum, FAC (where applicable), and Fibronectin HiLyte 488 (Fn-488; Cytoskeleton) at 4 μg/ml (as described in Torr et al., 2015). ARP101 (Bio-Technne, Oxford, United Kingdom) was used to inhibit MMP2 in FAC-treated HDFs. HDFs were incubated for 24 hours at 37 °C and 5% carbon dioxide and fixed and imaged.
on a confocal microscope (previously described). Remodeling of the 488-conjugated fibronectin was assessed through fluorescence intensity analysis in ImageJ software, version 1.8.0.

Zymography
Media, collected from HDFs cultured for 11 days, was loaded into 10% (v/v) acrylamide gels containing 0.2% (w/v) porcine skin gelatin (Thermo Fisher Scientific). Gels were resolved, stained, and quantified, as previously described (Wilkinson et al., 2018).

Fibronectin degradation
To determine whether MMP2 degraded fibronectin, human fibronectin (Sigma-Aldrich) was incubated in recombinant MMP2 (Bio-Techne) for 24 hours, with or without 1 mM APMA (Sigma-Aldrich) and 20 μM ARP101. Fibronectin was assessed by western blot, using mouse anti-fibronectin antibody (as described previously).

Scanning electron microscopy
HDFs were cultured on coverslips with FAC for 11 days, fixed in 2.5% glutaraldehyde (in distilled water) for 2 hours and rinsed in Dulbecco’s phosphate-buffered saline to remove glutaraldehyde. Coverslips were dehydrated in ethanol prior to critical point drying with carbon dioxide. Coverslips were then mounted onto pegs and sputter coated in carbon before imaging on a Zeiss EVO60 scanning electron microscope (Carl Zeiss).

SUPPLEMENTARY REFERENCES

Supplementary Figure S1. Diabetic wounds show altered collagen deposition and transcription during late-stage wound repair. (a)
Representative normal wound images for collagen I with provided regions (white boxes): NDb and Db wounds at (b) ×20 and (c, collagen I) ×2.5 magnification. qRT-PCR of Fn1, Col3a1 and Col1a1 in (d) normal and (e) NDb versus Db wounds. *P < 0.05, **P < 0.01, red *P < 0.001. D0, normal skin; D1, day 1 post wounding; D3, day 3 post wounding; D7, day 7 post wounding; Db, diabetic; NDb, nondiabetic; qRT-PCR, quantitative real-time reverse transcriptase–PCR. Magnification scale: ×2.5 bar = 200 μm; ×20 bar = 50 μm.
Supplementary Figure S2. Picrosirius red staining demonstrates altered dermal matrix in diabetic wounds. D0 and wounds at D1, D3, D7, and D14 were collected from wild-type mice (n = 5 per group). (a) Picrosirius red staining showed changes in early (green-yellow birefringence, arrows) and late (red-orange birefringence, arrows) ECM and (b) quantified. (c, images) NDb and Db staining, showing delayed ECM deposition in the Db group and (d) quantification. Mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. D0, normal skin; D1, day 1 post wounding; D3, day 3 post wounding; D7, day 7 post wounding; Db, diabetic; ECM, extracellular matrix; NDb, nondiabetic; SEM, standard error of the mean. Bar = 50 μm.
Supplementary Figure S3. Ferric ammonium citrate treatment alters MMP2 and collagen via cell-mediated responses. (a) Western blot demonstrates MMP2 degradation of fibronectin, rescued by ARP101. (b) MMP2 and (c) MMP9 standards treated with (d, quantified) FAC to demonstrate that iron does not directly affect protease activity. Black squares on gels depict bands measured. Full FAC dose experiment for (e) collagen III and (f) collagen I staining including DEF (deferoxamine plus 100 μM FAC), VEH (PMA vehicle), and PMA (non-iron oxidant). FAC = μM concentration. n = 3 donors. Mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. APMA, MMP2 activator; FAC, ferric ammonium citrate; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean.
Supplementary Figure S4. Iron causes oxidative stress induced collagen deposition, which is attenuated by mannitol and STEAP3 siRNA. 

(a, b) CellROX assay full data set for FAC, including DEF (deferoxamine plus 100 μM FAC). 
(c) Fold gene expression from qRT-PCR experiments comparing normal skin (D0) with wounds in wild-type mice (left panel) and diabetic with nondiabetic skin and wounds (right panel). Fold change below 5 = saturated blue color. 
(d, e) MAN treatment reduces ferritin. 
(e) Confirmation of STEAP3 siRNA. 
(f, g) STEAP3 siRNA reduces collagen III. Mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. D1, day 1 post wounding; D3, day 3 post wounding; D7, day 7 post wounding; MAN, mannitol; qRT-PCR, quantitative real-time reverse transcriptase–PCR; SEM, standard error of the mean; si-Ctrl, control siRNA; siRNA, small interfering RNA; si-S3, STEAP3 siRNA. Bar = 50 μm.
**Supplementary Table S1. Primer Sequences for all qRT-PCR Experiments**

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<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>GGCATGGACTGTGTCATGAG</td>
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<td>GAGCAAGGAAGCAACCCAG</td>
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Abbreviation: qRT-PCR, quantitative real-time reverse transcriptase–PCR