Title: Reduced iron in diabetic wounds: An oxidative stress-dependent role for

STEAP3 in extracellular matrix deposition and remodelling

Running Title: Iron modulates dermal wound matrix

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Abbreviations: Db (Diabetic), ECM (extracellular matrix), HDF (human dermal fibroblast), ICP-MS (inductively coupled plasma mass spectrometry), ICP-OES (ICP optical emission spectrometry), FAC (ferric ammonium citrate).

ABSTRACT

Iron is crucial for maintaining normal bodily function, with well-documented roles in erythropoiesis, haemostasis and inflammation. Despite this, little is known about the temporal regulation of iron during wound healing, nor how iron contributes to wound biology and pathology. Here we profiled tissue iron levels across a healing time-course, identifying iron accumulation during late-stage repair. Interestingly, 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 deposition and remodelling 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 to our knowledge previously unreported mechanistic role for iron in facilitating the remodelling stage of wound healing. Indeed, targeting tissue iron may be a promising future strategy to tackle the development and progression of chronic wounds.

Key Words: Iron, Wound Healing, Oxidative Stress, Extracellular Matrix.

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, where 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 (Fe^{2+}), iron reduces O_2 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 (Fe^{3+}) is exported into the bloodstream, where it binds mainly to transferrin (Tf). Tf-Fe then binds to transferrin receptor 1 (TfR1) at the cell surface (Arezes and Nemeth, 2015), the Tf-TfR1 complex is internalised via clathrin-mediated endocytosis, and Fe^{3+} is released (Mayle et al, 2012). Fe^{3+} is reduced to Fe^{2+} via the ferrireductase, STEAP3 (six-transmembrane epithelial antigen of prostate), with Fe^{2+} transported into the cytoplasm by divalent metal transporter 1 (DMT1; 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 (reviewed in Arezes and Nemeth, 2015), in turn linked to chronic pathologies (e.g. cardiovascular disease, Qi et al, 2007; 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 II diabetes mellitus (T2DM) in humans (Eshak et al, 2018). The effects of iron in mediating diabetes risk are further shown in the iron-loading disorder hereditary hemochromatosis (HH), 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 (reviewed in Barton and Acton, 2017; McClain et al, 2006). Thus, it is clear that altered iron metabolism mediates many of the risks factors for the development of T2DM.

In wound healing, iron is known for its oxidative role in haemostasis, where ferrous iron is released from haemoglobin (Lipinski and Pretorius, 2012) and acts to promote blood clotting (Kell and Pretorius, 2015; Pretorius et al, 2013). Abundant wound heme also directs inflammation through the release of hydroxyl radicals (Yeoh-Ellerton and Stacey, 2003), influences monocyte differentiation into macrophages (Haldar et al, 2014), and Fe^{2+} itself retains effects on macrophage function and polarisation (e.g. Kroner et al, 2014; Sindrilaru et al, 2011; Sindrilaru and Scharfetter-Kochanek, 2013; Agoro et al, 2018). Iron-dependent enzymes are also required for stimulating angiogenesis (Loenarz and Schofield, 2008; Ozer and Bruick, 2007) and are involved in the post-translational stabilisation 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 this links to pathological healing.

In the present study, we report temporal changes in endogenous iron distribution during normal acute wound healing and across a pathological murine model. We determine a specific, to our knowledge previously unreported, role for iron in aiding extracellular matrix (ECM) remodelling 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 remodelling phase of wound healing. ICP-MS, the gold standard technique for measuring metal abundance (Lee et al, 2014), was used to characterise tissue iron concentration across a normal healing time-course. Wounds were evaluated at days 1, 3, 7 and 14 post-wounding (D1, D3, D7 and D14 PW) in C57/Bl6 mice, with iron abundance compared to unwounded skin (D0; **Figure 1a**). A substantial increase in tissue iron concentration was observed at D7 and D14 PW, compared to D0 ($P < 0.001$). Perl's Prussian blue (PPB) histological staining independently confirmed the ICP-MS data (**Figure 1b**), where quantification (**Figure 1c**) showed increased iron deposition at D7 ($P < 0.001$) and D14 ($P < 0.001$) PW. The deposition of iron at D7 correlated temporally with wound collagen production (**Figure 1d-f**; **Supplementary Figure 1a**) and increased *Col3a1* ($P < 0.01$) and *Col1a1* ($P < 0.001$) mRNA (**Supplementary Figure 1d**).

Diabetic wound pathology is characterised by impaired iron accretion and reduced ECM deposition. We next asked whether delayed healing diabetic wounds (Wilkinson et al, 2019) displayed altered local iron levels. Specifically, ICP-MS/ICP-OES was used to evaluate iron abundance in non-diabetic (NDb) and diabetic (Db) skin (D0) and wounds at D3 and D7 PW (**Figure 2a**). As in wild-type mice (**Figure 1a**), NDb wounds displayed significantly elevated iron at D7 ($P < 0.01$, versus D0). Crucially, at all time-points, Db skin ($P < 0.001$) and wounds (D3, $P < 0.01$; D7, $P < 0.001$) presented with significantly reduced iron compared to NDb. Once again PPB histology confirmed metal profiling data, showing reduced iron deposition in the Db model at D3, D7 and D14 ($P < 0.001$; **Figure 2b-c**).

In line with observed iron changes, Db wounds displayed reduced collagen deposition at D7 PW (**Figure 2d-g**; **Supplementary Figure 1b-c**) and impaired *Col3a1* and *Col1a1* transcription (**Supplementary Figure S1e**). Indeed, immature/mature ECM fibre analysis (picrosirius red) confirmed reduced mature fibres in Db wounds (**Supplementary Figure S2**). Collectively, these data highlight strong correlation between wound tissue iron levels and deposition of wound extracellular matrix.

Iron administration increases intracellular iron storage. In order to evaluate the direct effects of iron on ECM production and deposition, we moved to 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 non-cytotoxic (**Figure 3b**). A ferrozine assay (Riemer et al, 2004) directly quantified 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 ($P < 0.05$; D5; **Figure 3d**) and immunocytochemistry ($P < 0.001$; **Figure 3e-f**). Thus, it is clear that HDFs internalise and store iron from exogenously administered FAC.

FAC treatment of HDFs dose-dependently increases fibronectin remodelling. Interestingly, 100 µM FAC led to reduced endogenous fibronectin deposition compared to 10 µM FAC ($P < 0.01$; **Figure 4a-b**). We next performed an Fn-488 assay to assess the influence of FAC on the remodelling of exogenous fibronectin. HDFs treated with 100 µM FAC for 24 hours demonstrated substantially increased remodelling ($P < 0.01$; increased 488 intensity), while treatment with ARP101, an inhibitor of matrix metalloproteinase 2 (MMP2), significantly reduced remodelling in the presence of FAC ($P < 0.01$; **Figure c-d**). As MMP2 inhibition reduced fibronectin remodelling, MMP2 activity in HDFs was assessed via gelatin zymography. Here, FAC treatment

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significantly increased activity of the protease MMP2 ($P < 0.05$; **Figure 4f-g**). Confirmation that MMP2 degrades fibronectin is provided in **Supplementary Figure S3a**. Zymography performed on MMP2 and MMP9 standards incubated with FAC excludes direct inhibition of MMP activity (**Supplementary Figure S3b-d**). Returning to 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 MMP able to remodel fibronectin *in vitro*.

FAC administration accelerates extracellular deposition of type I and type III collagen. Hydroxyproline assay was used to assess collagen content in HDFs treated with FAC. Here, significantly increased hydroxyproline was observed following 100 µM FAC treatment ($P < 0.001$; **Figure 4g**). This was confirmed by immunocytochemistry, where 100 µM FAC significantly increased the deposition of collagen III ($P < 0.01$) and collagen I ($P < 0.001$) in HDFs *in vitro* (D11; **Figure 4h-i**). These assays were also performed on a range of FAC treatments, where we saw a dose-dependent increase in collagen. Co-treatment with the iron chelator deferoxamine attenuated this increase in collagen, while the non-iron oxidant (phorbol 12-myristate 13-acetate, PMA) failed to increase collagen levels, demonstrating an iron-specific effect (**Supplementary Figure S3e-f**). Finally, SEM allowed determination of structural changes following FAC treatment at higher resolution than confocal microscopy. Intriguingly, 100 µM FAC treatment led to large microvesicles and increased extracellular secretion versus the control group (**Figure 4j**). Together, these data indicate that iron administration leads to significant extracellular matrix 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 showed that FAC treatment increased oxidative stress in HDFs, which was attenuated by deferoxamine ($P < 0.01$; **Supplementary Figure S4a-b**), and the antioxidant, mannitol ($P < 0.05$, **Figure 5a-b**). Interestingly, mannitol treatment also reduced the FAC-mediated secretion of collagen III ($P < 0.001$, **Figure 5a, c**) and collagen I ($P < 0.01$, **Figure 5a, 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**). Here, *Steap3* was identified as elevated in D7 WT wounds ($P < 0.05$; **Figure 5e**), and decreased in D7 Db wounds ($P < 0.01$; **Figure 5f**). *STEAP3* was significantly increased in HDFs following 100 µM FAC administration (D3; $P < 0.05$, **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 with the antioxidant mannitol attenuated *STEAP3* expression ($P < 0.05$; **Figure 5h**) and reduced HDF ferritin stores ($P < 0.001$, **Supplementary Figure S4d**). Next, siRNA was used to target *STEAP3* in HDFs and assessed its role in iron-induced collagen production. qRT-PCR demonstrated significant, stable knockdown of *STEAP3* (**Supplementary Figure S4e**). In HDFs treated with *STEAP3* siRNA, extracellular production of collagen III (**Figure 5i-j**) and collagen I (**Supplementary Figure S4f-g**) was significantly reduced following 100 µM FAC (compared to control siRNA). Finally, siRNA targeting *STEAP3* led to reduced oxidative stress in the presence of 100 µM FAC ($P < 0.001$; **Figure 5k**). Together, these data suggest that FAC-induced oxidative stress mediates collagen deposition, in part, through *STEAP3*.

DISCUSSION

Iron is a vital trace element, essential for life. Its role as a redox active metal is well-known, as is its major function in erythropoiesis (Majmundar et al, 2010). Here, we provide to our knowledge previously unreported findings showing the temporal regulation of iron during murine wound healing, and reveal its impairment in diabetic wound pathology. To date, iron has been largely recognised for driving haemostasis (e.g. Lipinski and Pretorius, 2012) and modulating inflammatory cell behaviour (Kroner et al, 2014; Agoro et al, 2018) during early-stage wound healing processes. Our data now demonstrate that iron plays a major role in modulating dermal extracellular matrix deposition and remodelling that characterises late-stage wound repair (Young and McNaught, 2011). We note that previous groups have measured iron in experimental rat wounds (using flame atomic absorption spectroscopy, Lansdown et al, 1999), and in chronic wound exudate (ICP-optical emission spectroscopy, Yeoh-Ellerton and Stacey, 2003). However, to our knowledge, this is the first time that endogenous iron has been compared 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 clearly stimulates HDFs to increase MMP2 expression, which may contribute to remodelling of fibronectin *in vitro*. Indeed, MMP2 has previously been shown to successfully cleave fibronectin (Jiao et al, 2012), an ECM component required during haemostasis and granulation tissue formation for epithelial migration and cellular adhesion (Barker and Engler, 2017; Leiss et al, 2008).

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Despite its crucial roles in early repair, stronger and more stable collagen fibres replace fibronectin during dermal remodelling (reviewed in Lenselink, 2013). Our data demonstrates that iron strongly stimulates extracellular deposition of both collagen type III and collagen type I *in vitro*. In HH, patients show local inflammation and fibrosis (Wang et al, 2017; reviewed in Wood et al, 2008), although this is most likely an indirect result of comorbities 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, given the widely reported gender differences in healing (Gilliver et al., 2008), future studies could assess gender 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 will be to confirm the role of iron in human Db wound pathology.

Intriguingly, iron delivery *in vitro* aids spheroid growth and ECM production (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 (Pourcelot et al, 2015). As a potential mechanism, iron loading is also known to induce cellular oxidative stress through the release of hydroxyl radicals (Gao et al, 2009). In turn, H_2O_2-induced oxidative stress promotes collagen production in cardiac rat 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 TGFβ-induced murine fibrosis models (Anathy et al, 2018). Finally, systemic sclerosis (Ssc) skin fibroblasts (from patients and mice), which possess excessive

dermal ECM, are characterised by heightened ROS (Sambo et al, 2001; Kavian et al, 2010), which can be downregulated by the antioxidant stimulator, nuclear factor-like 2 (Kavian et al, 2018). Thus, we and others show 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 D7 PW, yet Db wounds displayed an altered profile of iron gene expression, with significant downregulation of *Steap3* compared to NDb wounds. In fact, targeting of *STEAP3*, which is required for iron transport into the cytoplasm (via DMT1, Ohgami et al, 2005), led to 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 organisation. 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 iron in murine pathological healing. Of therapeutic interest, endosomal conversion of exogenously administered iron via *STEAP3* may accelerate ECM deposition through an oxidative stress-dependent mechanism. Further studies, particularly using human tissue, are now essential to explore the feasibility of directly manipulating tissue iron levels to promote wound healing.

MATERIALS AND METHODS

Animal Experimentation: Mice, purchased from Envigo Ltd. (UK), were housed at the Biological Services Facility at The University of Manchester (UK) with *ad libitum*

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access to food and water. All animal experimentation was performed according to UK Home Office regulations under project licence 70/8136.

Wounding Experiments: Female WT (wild-type) C57/Bl6, NDb (non-diabetic; $Lepr^{-/+}$) and Db (diabetic; $Lepr^{-/-}$) mice were anaesthetised and wounded at 8-10 weeks old using our established protocol (Ashcroft and Mills, 2002) with modifications. Here, two equidistant 6mm excisional wounds were created with trace metal free (TMF) titanium instruments (World Precision instruments, Hertfordshire, UK) on the dorsum of each mouse. Mice were administered buprenorphine post-operatively. For WT time course experiments, wounds were collected at days one (D1), three (D3), seven (D7) and fourteen (D14) post-wounding (PW). For NDb vs Db time course experiments, wounds were collected at D3 and D7 PW only. Normal skin (D0) was also collected. Further details are provided in the supplementary text.

Metal Quantification: The samples were analysed by a combination of ICP-MS, one of the most sensitive, commercially available techniques for element analysis (Liu et al, 2014), and ICP-OES (ICP-optical emission spectrometry). Tissue frozen at -80°C was freeze dried in TMF vacutainers (BD Biosciences, Wokingham, UK) 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) were prepared in TMF nitric acid (HNO_3, Sigma-Aldrich, Dorset, UK) and 30% hydrogen peroxide (H_2O_2, Sigma-Aldrich) as in Ouypornkochagorn and Feldmann (2010) and digested as described in the supplementary text. Samples were subsequently analysed on an Agilent 7500cx inductively coupled plasma mass spectrometer (Agilent Technologies, Cheshire, UK) or Optima 5300DV inductively coupled plasma optical emission spectrometer (PerkinElmer, Buckinghamshire, UK).

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Histological Analysis: Tissue samples were stained with Perl's Prussian blue (PPB) to detect iron deposits. Immunohistochemistry was performed using mouse anti-fibronectin (clone EP5, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-collagen III (ab7778) and rabbit anti-collagen I (clone EPR7785, Abcam, Cambridge, UK) primary antibodies. Further details are provided in the supplementary text.

Quantitative Real-Time PCR (qRT-PCR): RNA was extracted from wounds, normal skin tissue and cells. Full information for qRT-PCR experiments are in the supplementary text with primer sequences provided in **Supplementary Table S1**.

Fibroblast Culture: Primary human dermal fibroblasts (HDFs) were isolated from human skin from donors < 50 years of age, obtained with institutional approval and full, written informed consent from Castle Hill Hospital, Hull, UK (17/SC/0220) as previously described (Wilkinson et al, in press). HDFs were cultured in MEM (Minimum Essential Media, Thermo Fisher Scientific, Loughborough, UK) containing 10% FBS (foetal bovine serum) and 1% P/S (penicillin-streptomycin solution, both Thermo Fisher Scientific). FAC was used as described in the supplementary text. Growth and viability with FAC treatment were determined. ECM production was assessed via immunocytochemistry, western blotting and scanning electron microscopy as described in the supplementary text.

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 manufacturer's instructions. After 6 hours, media was replaced with fresh antibiotic-free MEM containing 2% FBS and HDFs were treated with FAC

for 5 days and analysed (qRT-PCR, immunocytochemistry and oxidative stress). Silencer® Select Negative Control No. 1 SiRNA was used as a validated control.

Oxidative Stress Experiments: Oxidative stress was measured in HDFs following FAC treatment using CellROX™ Green Reagent (Thermo Fisher Scientific). HDFs were imaged via confocal microscopy (LSM 710, Carl Zeiss Ltd., Cambridge, UK) at 20X magnification and intensity of staining (CTCF, corrected total cell fluorescence) was determined (McCloy et al, 2014). To inhibit oxidative stress, mannitol (Thermo Fisher Scientific) was added at the time of FAC administration.

Statistical Analysis: Mean +/- standard deviations of the mean (SEM) were used for all data sets. Statistical analyses were performed on all quantitative data using GraphPad Prism 7 (GraphPad Software, California, US). Independent t tests, one-way ANOVA and two-way ANOVAs were used, with *post-hoc* analyses (Dunnett's and Tukey) performed on significant ANOVAs. Significance between experimental groups was accepted when $P < 0.05$.

DATA AVAILABILITY STATEMENT

No datasets were generated or analyzed during the current study.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS STATEMENT

The authors contributed in the following ways: study conceptualisation, project administration, and methodology: HW and MH; investigation and validation: HW, SU, KB, RK; formal analysis, visualisation, and manuscript preparation: HW. Manuscript revision: HW, KM and MH; resources: KM and MH; acquisition of funding and study supervision: MH.

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FIGURE LEGENDS

Figure 1. Wound iron abundance increases during normal repair and temporally correlates with collagen deposition. Iron abundance increased at day 7 (D7) and D14 post-wounding in normal mice (**a**, n=5 per group), confirmed via Perl's Prussian Blue (PPB) staining (**b**), quantified in **c**. Arrows = deposits. Spleen = PPB positive control. Immunocytochemistry staining (**d**) and quantification for fibronectin (**e**), collagen III (**f**) and collagen I (**g**). DAPI = counterstain. Bars = 50 μm. Mean +/- SEM. * = $P < 0.05$, ** = $P < 0.001$, red * = $P < 0.001$.

Figure 2. Diabetic wounds display impaired iron accumulation and delayed collagen deposition. Diabetic (Db) skin and wounds possessed significantly less iron than non-Db (NDb; **a**; n=5 per group). # = NDb versus Db. PPB staining (**b**, quantification in **c**). Arrows = deposits. Spleen = PPB positive control. Immunocytochemistry staining (**d**) for fibronectin (**e**), collagen III (**f**) and collagen I (**g**). D0 = normal skin. D3 = day 3 post-wounding. DAPI = counterstain. Bar = 50 μm. Mean +/- SEM. * = $P < 0.05$, ** = $P < 0.001$, red * or # = $P < 0.001$.

Figure 3. Human dermal fibroblasts sequester administered iron. Human dermal fibroblasts (HDFs) growth kinematics (**a**) and viability (**b**) following ferric ammonium citrate (FAC) treatment. PI = propidium iodide. EtOH = ethanol control. Sequestration of FAC determined by ferrozine (**c**), western blot (**d**) and immunocytochemistry (**e**, quantified in **f**). Ferritin statistics versus 100 μm FAC. DEF = deferoxamine iron chelator plus 100 μm FAC. Ferritin = red. DAPI = counterstain. Arrows = ferritin. Bars = 50 μm. CTCF = corrected total cell fluorescence. n=3 donors. Mean +/- SEM. * = $P < 0.05$, ** = $P < 0.001$, red * = $P < 0.001$.

Figure 4. Iron administration causes fibronectin remodelling and accelerated collagen deposition *in vitro*. Human dermal fibroblasts (HDFs) treated with FAC (ferric ammonium citrate, 11 days) and stained for fibronectin (green, **a-b**). Fn-488 remodelling

(**c**, images, **d**). ARP101 MMP2 inhibitor groups plus 100 μM FAC. Zymography (**e**). A = control, B = 10 μM FAC, C = 100 μM FAC. *MMP2* qRT-PCR (**f**). Hydroxyproline (**g**) and collagen type III (COL III) and I immunocytochemistry (**h**, quantification, **i**). Collagens = green. Bars = 50 μm. Scanning electron microscopy (**j**). Secretions = grey arrows. Microvesicles = white arrows. Bars = 10 μm. Inset bars = 2.5 μm. n=3 donors. Mean +/- SEM. * = $P < 0.05$, ** = $P < 0.01$, red * = $P < 0.001$.

Figure 5. Intracellular iron loading accelerates collagen deposition via oxidative stress-dependent upregulation of *STEAP3*. Ferric ammonium citrate (FAC) increased oxidative stress, inhibited by mannitol (MAN; **a**, quantified, **b**). CellROX® 488 = oxidative stress, phalloidin (red) and DAPI (blue). Collagen III (**c**) and I (**d**) deposition. *Steap3* qRT-PCR in normal (**e**) and diabetic (Db) wounds (**f**). D0 = skin. D7 = day 7 post-wounding. *STEAP3* qRT-PCR in human dermal fibroblasts (**g**), which is inhibited by MAN (**h**). *STEAP3* siRNA (si-S3) reduced collagen I (**i** and **j**) and oxidative stress (**k**). si-Ctrl = control siRNA. CTCF = corrected total cell fluorescence. Bars = 50 μm. n=3 donors. Mean +/- SEM. * = $P < 0.05$, ** = $P < 0.01$, red * = $P < 0.001$.

SUPPLEMENTARY MATERIAL