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Local Arginase 1 Activity Is Required for Cutaneous Wound Healing

Laura Campbell[1], Charis R. Saville[1], Peter J. Murray[2], Sheena M. Cruickshank[3,4] and Matthew J. Hardman[1,4]

Chronic nonhealing wounds in the elderly population are associated with a prolonged and excessive inflammatory response, which is widely hypothesized to impede healing. Previous studies have linked alterations in local L-arginine metabolism, principally mediated by the enzymes arginase (Arg) and inducible nitric oxide synthase (iNOS), to pathological wound healing. Over subsequent years, interest in Arg/iNOS has focused on the classical versus alternatively activated (M1/M2) macrophage paradigm. Although the role of iNOS during healing has been studied, Arg contribution to healing remains unclear. Here, we report that Arg is dynamically regulated during acute wound healing. Pharmacological inhibition of local Arg activity directly perturbed healing, as did Tie2-cre-mediated deletion of *Arg1*, revealing the importance of Arg1 during healing. Inhibition or depletion of Arg did not alter alternatively activated macrophage numbers but instead was associated with increased inflammation, including increased influx of $iNOS^+$ cells and defects in matrix deposition. Finally, we reveal that in preclinical murine models reduced Arg expression directly correlates with delayed healing, and as such may represent an important future therapeutic target.

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INTRODUCTION

Expanding global elderly and diabetic populations combined with a continued lack of effective treatment modalities means the incidence of chronic wounds is increasing. Chronic wounds are associated with an excessive inflammatory response, which is widely accepted to be a major causative factor in the multifactorial healing pathology (Loots *et al.*, 1998; Diegelmann, 2003). Macrophages, the key mediators of the inflammatory response to infection and repair, display clear plasticity that permits development into a spectrum of phenotypes depending on environmental and cytokine signals. Seminal studies have classified the major macrophage subtypes that lie at the polar ends of the spectrum, these include (a) Th1-induced classically activated macrophages (CAMs)—IFN-γ and tumor necrosis factor-α induced with enhanced antimicrobial capacity and proinflammatory cytokine production (Mosser and Zhang, 2008) and (b) Th2-induced alternatively activated macrophages (AAMs)—IL-4 and/or IL-13 induced with anti-inflammatory ''tissue'' repair functions (Gordon and Martinez, 2010). Although the disease relevance of macrophage polarization (or lack of) has been demonstrated in numerous tissue pathologies and clearly linked to disease progression (Hesse *et al.*, 2001; Pesce *et al.*, 2009; Sindrilaru *et al.*, 2011), the contribution of these macrophage subtypes to chronic wound pathology remains unclear.

CAMs and AAMs are phenotypically different with AAMs identified through the expression of cell surface receptors IL4Rα chain and mannose receptor and intracellular enzymes *Retnla* (encoding Fizz1/RELMα), *Chi3l3* (Ym1), and *Arg1* (Gordon and Martinez, 2010). CAMs and AAMs are thought to have different functions during the host response, mediated partly by the upregulation of intracellular enzymes, inducible nitric oxide synthase (iNOS) in CAMs, and arginase (Arg) in AAMs. Although Arg1 is predominantly associated with AAMs, its expression has also been observed in CAMs in chronic parasitic and bacterial infection (El Kasmi *et al.*, 2008; Gordon and Martinez, 2010). Interestingly, iNOS and Arg can compete for their common substrate, the amino acid L-arginine, which is a key component of the urea cycle. L-arginine metabolism by iNOS, through substrate competition with Arg, produces L-citrulline and nitric oxide, a critical mediator of immunological and physiological aspects of tissue repair. It is noteworthy that iNOS-deficient mice display altered epithelial and endothelial cell proliferation and migration (Ziche *et al.*, 1994; Yamasaki *et al.*, 1998). Arg exists as two isoforms with Arg1 previously linked to tissue regeneration (Peranzoni *et al.*, 2007). Both Arg1 and Arg2 metabolize L-arginine into L-ornithine and urea. L-ornithine is a

[1]*The Healing Foundation Centre, Faculty of Life Sciences, The University of Manchester, Manchester, UK;* [2]*Departments of Infectious Diseases and Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA and* [3]*Manchester Immunology Group, Faculty of Life Sciences, The University of Manchester, Manchester, UK*

[4]*These authors contributed equally to this work.*

Correspondence: Matthew J. Hardman, The Healing Foundation Centre, Faculty of Life Sciences, The University of Manchester, AV Hill Building, Oxford Road, Manchester M13 9PT, UK.
E-mail: matthew.j.hardman@manchester.ac.uk

Abbreviation: AAM, alternatively activated macrophage; Arg, arginase; CAM, classically activated macrophage; iNOS, inducible nitric oxide synthase; nor-NOHA, N(omega)-hydroxy-nor-L-arginine

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precursor of proline and polyamines, which promote collagen synthesis and cell proliferation, respectively, key aspects of tissue regeneration (Jenkinson *et al.*, 1996; Witte *et al.*, 2002). The expression and activity of Arg and iNOS must therefore be tightly regulated to provide tissues with the appropriate biological mediators. Indeed, a dysregulated balance between the local iNOS and Arg activity has been suggested to promote chronic disease (Unal *et al.*, 2005; Maarsingh *et al.*, 2006; Naura et al., 2010; Redente et al., 2010) and potentially impair wound healing in elderly subjects (Childress *et al.*, 2008; Debats *et al.*, 2009).

Recent studies have begun to focus on the role of macrophage activation/polarization during healing, with Miao *et al.*, (2012), reporting altered macrophage activation in diabetic mouse wounds. Data in this area remain somewhat contentious with iNOS-deficient mice displaying delayed healing or no effect on healing depending on the wound model investigated (Yamasaki *et al.*, 1998; Most *et al.*, 2002). Surprisingly, although Arg has been found to be functionally important in multiple disease pathologies (Abeyakirthi *et al.*, 2010; Maarsingh *et al.*, 2006; Pesce *et al.*, 2009), little is known about the role of Arg1 in normal skin repair. Here, we report the effects of both functional Arg inhibition (via local nor-NOHA treatment) and genetic ablation of Arg1 (cell-specific deletion *T2C;Arg1*$^{fl/fl}$) during skin repair. In both models, Arg deficiency delays healing associated with an altered inflammatory response and abnormal matrix deposition.

RESULTS

Arg1 is dynamically regulated during acute healing

Previous studies have suggested that macrophage phenotype is temporally regulated during wound healing, with CAMs present at early stages and AAMs more dominant during later stages (Albina *et al.*, 1990; Daley *et al.*, 2010). We confirmed this temporal profile in our C57/Bl6 excisional wound model using immunohistochemistry for Arg1 and iNOS, widely accepted markers of CAM and AAM activation, respectively (Gordon and Martinez, 2010). In the acute healing model, iNOS levels peaked at 3 days post wounding, whereas Arg1 remained high until 7 days (Figure 1). These time points correlate with the transition from a proinflammatory extracellular milieu to a phase of matrix deposition (Shaw and Martin, 2009). To corroborate these findings further, we analyzed Arg enzymatic activity, which provides a

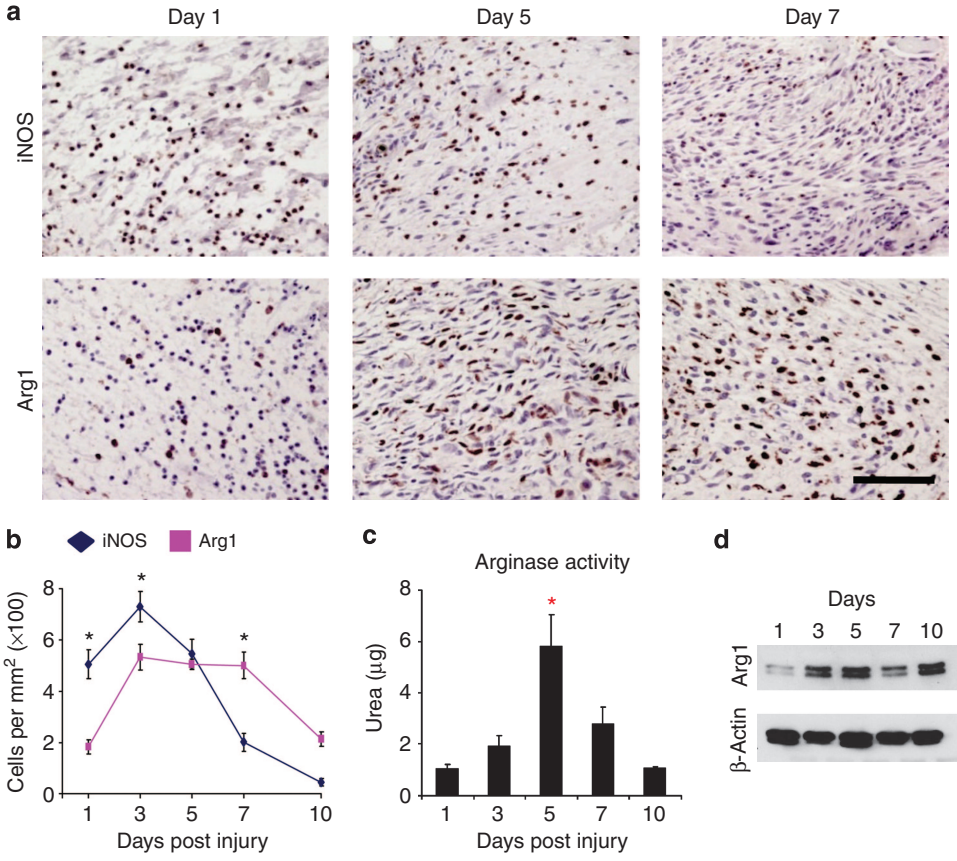

Figure 1. Arginase1 (Arg1) is dynamically regulated during healing. (**a**) Images representing the experimental group mean for inducible nitric oxide synthase-positive (iNOS$^+$) and Arg1$^+$ cells in 1-, 5-, and 7-day excisional wound granulation tissue. (**b**) Quantification of iNOS$^+$ and Arg1$^+$ dermal inflammatory cells reveals differing temporal profiles. Immunohistochemical quantification data are derived from the mean of five randomly selected high-powered fields per wound and two wounds per mouse. (**c**) Arginase activity from isolated excisional wound tissue (measured through urea production) peaks at 5 days post wounding. (**d**) Western blot analysis of total Arg1 protein in excisional wounds reveals increased expression at 3, 5, and 10 days post wounding. (**b**) Data presented indicate mean + SEM of $n = 5$–6 mice per group or (**c**, **d**) three replicates per group across two individual experiments. Bar = 100 μm. (**b**) *$P < 0.05$ comparing iNOS with Arg1, (**c**) *$P < 0.05$ compared with days 1, 3, and 10.

functionally relevant measure (Witte *et al.*, 2002). We report a strong peak in wound tissue Arg activity 5 days post wounding (Figure 1c), corresponding to the midpoint of the peak in wound granulation tissue Arg1$^+$ macrophages (Figure 1b). Finally, we profiled global Arg1 protein levels in isolated wound tissue over a healing time course. Total protein also peaked at day 5 in line with total tissue Arg activity (Figure 1d). It is to be noted that the subsequent increase in Arg1 at 10 days post wounding likely reflects the previously observed latter-stage induction in wound fibroblasts (Witte *et al.*, 2002).

Local inhibition of Arg activity significantly delays cutaneous healing

To explore the functional role of Arg during healing, a commonly used inhibitor of Arg activity, nor-NOHA (Tenu *et al.*, 1999; Takahashi *et al.*, 2010), was locally applied to incisional wounds. Nor-NOHA treatment significantly delayed healing, demonstrated by an increased histological wound area versus vehicle-treated wounds at both 3 and 7 days post wounding accompanied by reduced re-epithelialization (Figure 2a–c). Interestingly, delayed healing in nor-NOHA-treated wounds was accompanied by increased numbers of local macrophages (Figure 2e) maintained across days 3 and 7 post wounding. To determine the contribution of recruitment versus removal, we assessed local-wound chemokine levels and apoptosis. Delayed healing in nor-NOHA-treated wounds are associated with increased levels of inflammatory chemokines at 3 days post wounding with elevated apoptosis at 7 days post wounding (Supplementary Figure S1 online). Thus, pharmacological inhibition of Arg (Arg1 and Arg2) delays repair associated with altered local macrophage numbers. To confirm a specific role for Arg1, we next studied a cell-specific conditional *Arg1* knockout model.

Tie2-cre-mediated conditional ablation of *Arg1* (*T2C;Arg1$^{fl/fl}$*) reveals a cell-specific role for Arg during healing

Arg1 is thought to be expressed in multiple cell types involved in the healing process, including keratinocytes, inflammatory cells, and fibroblasts (Albina *et al.*, 1990; Witte *et al.*, 2002; Kampfer *et al.*, 2003). However, we hypothesized that macrophages would be the key Arg1-expressing cell type in the wound repair system. To test this idea, we used an Arg1 conditional allele crossed to Tie2-Cre, which is active in all hematopoietic and endothelial cells. As macrophages are the main Arg1-expressing cell type, Tie2-Cre deletion provides a convenient way to ablate macrophage Arg1, noting that some endothelial cells may also express the gene (El Kasmi *et al.*, 2008; Pesce *et al.*, 2009). Here, we report that Tie2-cre-mediated deletion of Arg1 (*T2C;Arg1$^{fl/fl}$*) resulted in a pronounced healing delay compared with *T2C;Arg1$^{+/+}$* littermate controls, depicted macroscopically at 3, 7, and 14 days post wounding (Figure 3a). Subsequent histological analysis revealed a substantial increase in wound area and reduction in re-epithelialization in *T2C;Arg1$^{fl/fl}$* wounds. This wound phenotype reveals an important role for macrophage/endothelial-derived Arg1 during skin repair (Figure 3b–d).

T2C;Arg1$^{fl/fl}$ mice exhibit alterations in wound inflammatory cell recruitment

An excessive inflammatory response is a common theme in pathological healing (Martin and Leibovich, 2005; Emmerson *et al.*, 2010). In light of the link of macrophage Arg1 to inhibition of inflammation, we assessed the inflammatory cell profile in delayed healing *T2C;Arg1$^{fl/fl}$* wounds. Tie2-cre-mediated Arg1 ablation led to an increased and extended influx of not only macrophages (Figure 4b and d) but also neutrophils (Figure 4a and c). We next assessed markers of macrophage polarization (Daley *et al.*, 2010). Interestingly, wound granulation tissue iNOS$^+$ cells (CAM marker) were increased in *T2C;Arg1$^{fl/fl}$* at both 3 and 7 days post wounding, suggesting a maintained pro-inflammatory environment (Figure 4e and g). These data were confirmed through increased nitrotyrosine staining (used as a marker of cell damage and inflammation through NO) in *T2C;Arg1$^{fl/fl}$* at both 3 and 7 days post wounding (data not shown). In contrast, no difference was observed in the number of wound cells expressing the AAM marker, Ym1, at either 3 or 7 days post wounding (Figure 4f and h). Thus, the macrophage component of the wound-healing phenotype in *T2C;Arg1$^{fl/fl}$* mice involves excessive numbers of iNOS$^+$ cells rather than a defect in the ability of macrophages to become polarized in the skin micro-environment. Intriguingly, previous studies have shown that isolated Arg-deficient macrophages have increased NO production in response to lipopolysaccharide stimulation (El Kasmi *et al.*, 2008; Pesce *et al.*, 2009). Crucially, our data suggest that despite Arg1 being associated with AAMs, Arg1-deficient macrophages remain able to adopt an AAM phenotype.

Excessive protease activity and reduced matrix deposition contribute to delayed healing in *T2C;Arg1$^{fl/fl}$* wounds

Arg-mediated L-arginine metabolism produces L-ornithine, which is an important component of collagen synthesis (Morris, 2009). We thus hypothesized that *T2C;Arg1$^{fl/fl}$* mice would display alterations in wound matrix deposition, synthesis, and/or remodeling. Indeed, delayed healing in *T2C;Arg1$^{fl/fl}$* mice was associated with reduced wound granulation tissue collagen deposition, assessed by collagen 1 immunofluorescence (Figure 5a) and substantially reduced total wound collagen 1 and 3 levels measured by western blot (Figure 5b). Conversely, at the level of gene expression, the major skin collagen species are increased in *T2C;Arg1$^{fl/fl}$* wounds. This fits with our previous studies, where delayed healing is phenotypically linked to increased collagen gene expression, presumably as a compensatory mechanism (Hardman *et al.*, 2005; Hardman and Ashcroft, 2008). Gelatinases (MMP2 and MMP9) have an important role in wound granulation tissue remodeling. However, excessive gelatinase activity has been linked to the reduced matrix deposition observed in chronic wounds (Lobmann *et al.*, 2002). Analysis of *T2C;Arg1$^{fl/fl}$* wounds at 7 days post wounding reveals a significant and selective increase in MMP2 at both the gene expression (Figure 5d) and protein (Figure 5e and f) levels.

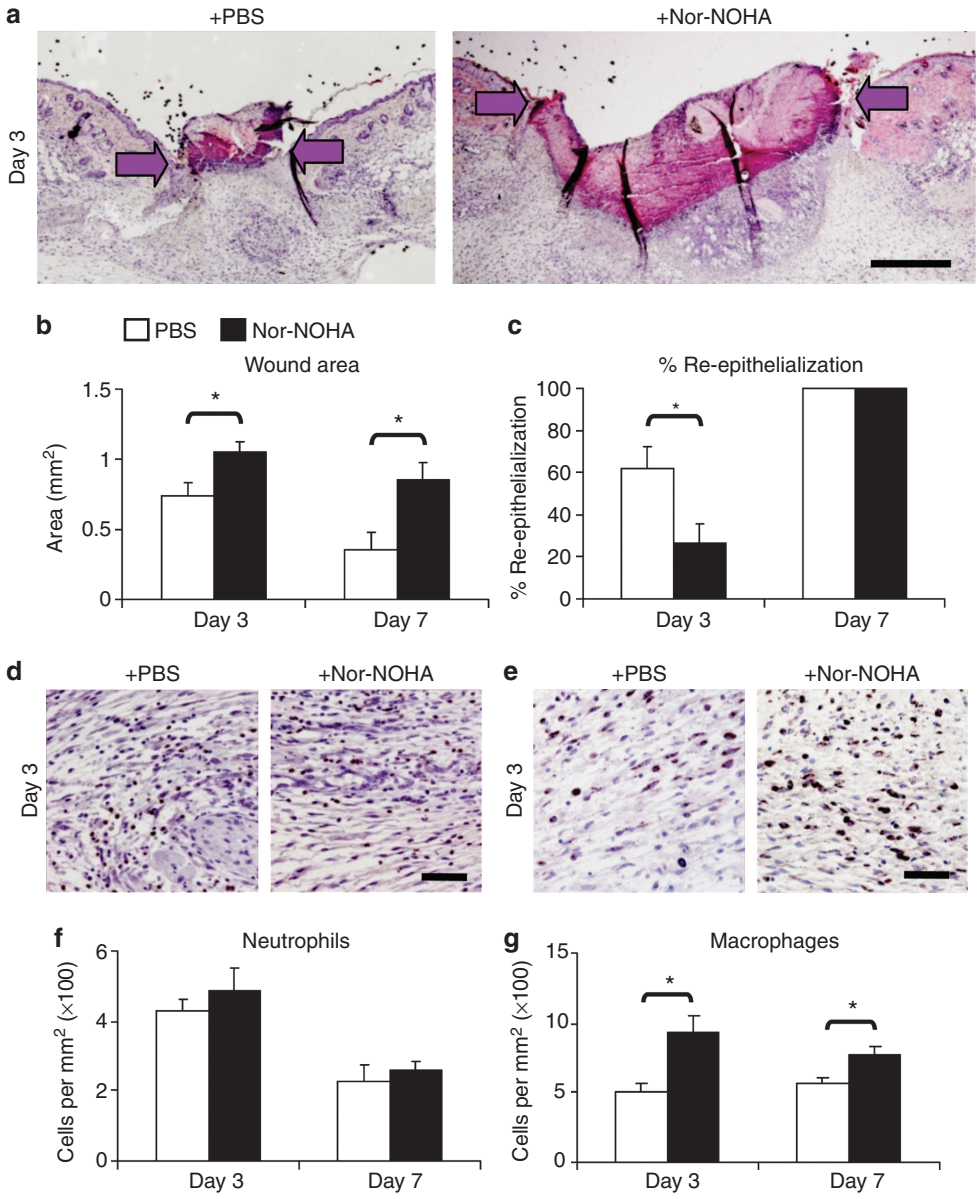

Figure 2. Inhibition of arginase activity significantly delays cutaneous healing. (**a**) Representative hematoxylin and eosin–stained incisional wounds (day 3) from control and N(omega)-hydroxy-nor-L-arginine (nor-NOHA)-treated mice. Arrows indicate wound margins. Nor-NOHA treatment significantly delays wound closure quantified by (**b**) increased wound area at 3 and 7 days post wounding and (**c**) decreased re-epithelialization at 3 days post wounding. Images representing the experimental group mean for (**d**) wound neutrophils and (**e**) macrophages from control and nor-NOHA-treated mice. (**f**) Quantification reveals no effect of nor-NOHA on wound neutrophil numbers. (**g**) Quantification of wound macrophage numbers reveals a significant increase following nor-NOHA treatment at 3 and 7 days post wounding. Immunohistochemical quantification data are derived from the mean of five randomly selected high-powered fields per wound and two wounds per mouse. Data presented indicate mean + SEM, $n = 6$ mice per group. Bar = 400 μm (**a**), 50 μm (**d**, **e**). *$P < 0.05$. PBS, phosphate-buffered saline.

Reduced Arg1 is a conserved feature of delayed-healing mouse wounds

Data presented thus far reveal that either pharmacological inhibition or genetic ablation of Arg *in vivo* leads to a significant delay in skin healing. To confirm the functional relevance to pathological healing, we turned to preclinical delayed-healing mouse models that have been extensively validated by our group and others (Hardman *et al.*, 2008; Holcomb *et al.*, 2009). We report significantly altered granulation tissue levels of iNOS$^+$ and Arg1$^+$ (Figure 6) cells in both aged and ovariectomized delayed-healing models. In both models, the levels of Arg1 was reduced, whereas that of iNOS was increased, presumably reflecting a delayed switch in macrophage polarization.

DISCUSSION

The contribution of macrophage polarization to cutaneous wound healing remains unclear. The situation is further

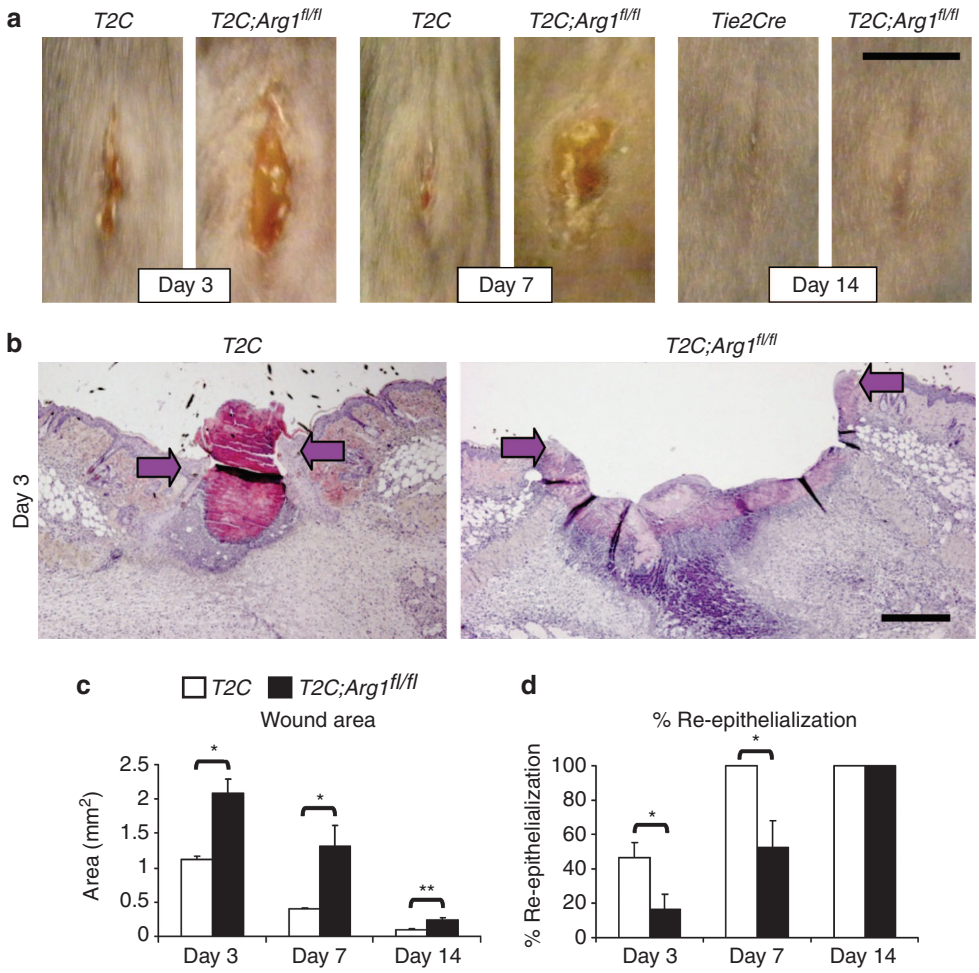

Figure 3. Conditional ablation of *Arg1* (*T2C;Arg1*$^{fl/fl}$) significantly delays cutaneous repair. (**a**) Representative macroscopic images of control (*T2C*) and *T2C;Arg1*$^{fl/fl}$ wounds at 3, 7, and 14 days post wounding show delayed healing in *T2C;Arg1*$^{fl/fl}$ wounds. (**b**) Representative hematoxylin and eosin–stained day-3 histology from *T2C* and *T2C;Arg1*$^{fl/fl}$ wounds. Arrows indicate wound margins. Dermal cell–specific deletion of arginase 1 significantly delays wound closure quantified by (**c**) increased wound area and (**d**) delayed re-epithelialization over multiple time points. Bar = 5 mm (**a**) and 400 μm (**b**). Mean + SEM, $n = 6–7$ mice per group. $^{**}P<0.01$, $^{*}P<0.05$.

complicated by the fact that markers of each macrophage phenotype, iNOS and Arg, compete for a common substrate, L-arginine, with products from each being important for healing (Childress *et al.*, 2008). Previous studies have shown that L-arginine administration is able to promote acute healing in both rodents and humans (Seifter *et al.*, 1978; Barbul *et al.*, 1990; Williams *et al.*, 2002), an effect attributed to increased nitric oxide production through iNOS metabolism of L-arginine (Shi *et al.*, 2000). Subsequent studies on the importance of iNOS are conflicting, depending largely on the wound model used (Yamasaki *et al.*, 1998; Most *et al.*, 2002). Data presented in this study reveal an important role of the Arg arm in the L-arginine metabolism pathway during cutaneous healing. We report similar findings across pharmacological inhibition and cell-specific genetic ablation studies, confirming relevance in preclinical delayed-healing mouse models.

A previous study reported accelerated acute wound healing following Arg inhibition with the compound (2)-(*S*)-amino-6-boronohexanoic acid (ABH) (Kavalukas *et al.*, 2011). The differing effects of Arg inhibition seen by Kavalukas *et al.*, and in this current study, are most likely due to the use of different inhibitors. This study used the compound nor-NOHA, which has been shown to be a potent inhibitor of Arg1 and Arg2 in a number of *in vitro/in vivo* models (Tenu *et al.*, 1999; Takahashi *et al.*, 2010). Kavalukas *et al.*, used the compound ABH, which is a more selective inhibitor of Arg2 compared with Arg1 *in vivo* (Baggio *et al.*, 1999). A previous study has shown that both Arg1 and Arg2 are expressed in multiple cutaneous cell types (e.g., keratinocytes and inflammatory cells) (Kampfer *et al.*, 2003). We confirmed the importance of Arg1 using *T2C;Arg1*$^{fl/fl}$ mice, which displayed an even more pronounced delayed-healing phenotype compared with that observed through nor-NOHA treatment. The clear correlation between these two independent models reinforces the importance of Arg1 during healing.

Arg1 is expressed across a range of cell types involved in wound healing including keratinocytes (Kampfer *et al.*, 2003),

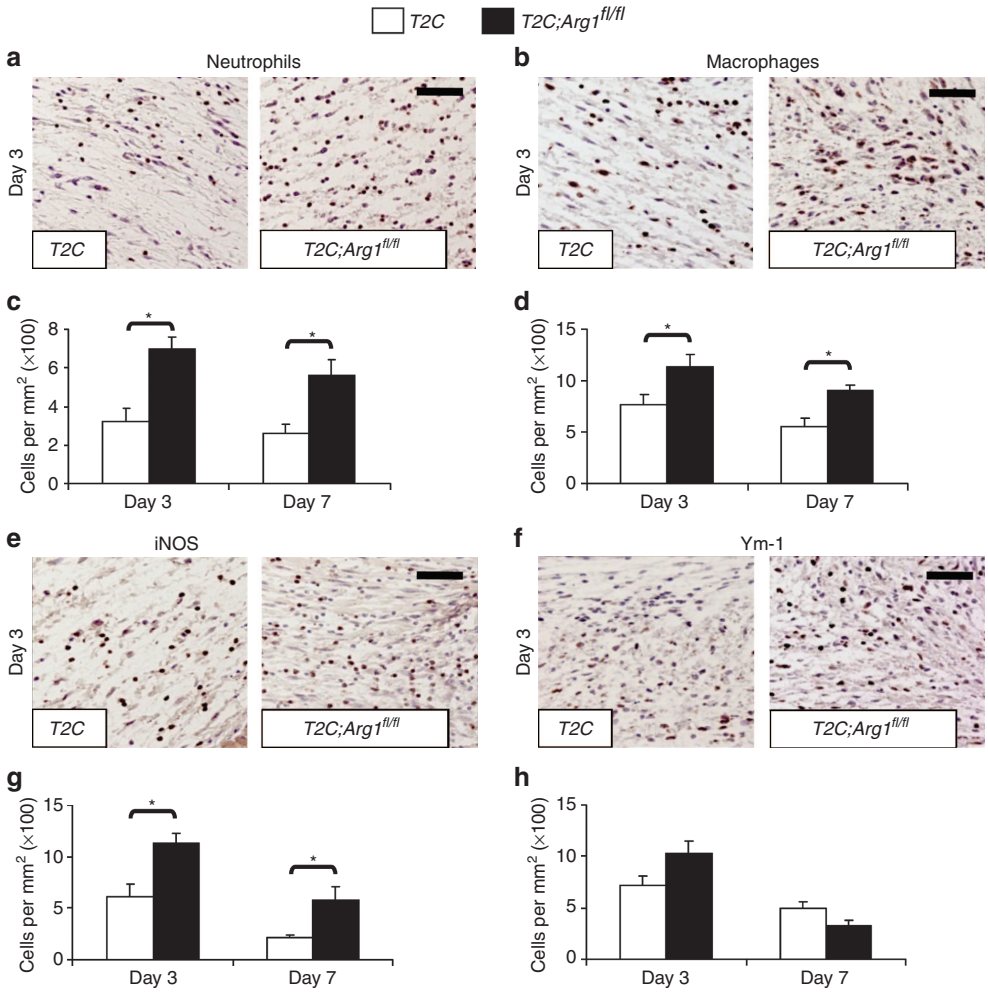

Figure 4. Conditional deletion of arginase 1 (Arg1) results in excessively prolonged and local inflammation. Images representing the experimental group mean for (**a**) neutrophil and (**b**) macrophage immunohistochemical analysis from day-3 wound granulation tissue. *T2C;Arg1*$^{fl/fl}$ wounds display significantly increased numbers of (**c**) neutrophils and (**d**) macrophages at 3 and 7 days post wounding. Images representing the experimental group mean for (**e**) inducible nitric oxide synthase-positive (iNOS$^+$) and (**f**) Ym1$^+$ cells. *T2C;Arg1*$^{fl/fl}$ wounds have increased numbers of (**g**) iNOS$^+$ cells, with no difference in (**h**) Ym1$^+$ cells. Immunohistochemical quantification data are derived from the mean of five randomly selected high-powered fields per wound and two wounds per mouse. Data presented indicate the mean + SEM of $n = 6–7$ mice per group. Bar = 50 μm. *$P < 0.05$.

fibroblasts (Witte *et al.*, 2002), endothelial cells (Abd-El-Aleem *et al.*, 2000), and inflammatory cells (Miao *et al.*, 2012). Here, we have used the *Tie2-cre* mouse to selectively ablate Arg1 in all hematopoietic and endothelial cell lineages (El Kasmi *et al.*, 2008). The fact that delayed healing in *Tie2cre;Arg*$^{fl/fl}$ mice is associated with alterations to a range of cell functions primarily attributed to additional cell types, e.g., re-epithelialization and matrix deposition, implies an important role for paracrine signaling. It is noteworthy that iNOS$^+$ cells are increased in *Tie2cre;Arg*$^{fl/fl}$ wounds in line with the proposed role of Arg1-expressing Th2-activated macrophages as suppressor cells that help to dampen Th1-driven inflammation (Pesce *et al.*, 2009). Indeed, this mechanism is most likely important in chronic wounds, which are widely accepted to be in a Th1 proinflammatory state (Sindrilaru *et al.*, 2011).

Arg1-mediated metabolism of L-arginine is an important source of local ornithine, a proline precursor important for collagen synthesis. However, the main cellular source of wound collagen is fibroblasts (Singer and Clark, 1999). Thus, the observed defect in collagen deposition following *Tie2-cre*-mediated deletion of *Arg1* is presumably a secondary effect of the delayed-healing environment. Indeed, the reduced collagen content of *Tie2cre;Arg*$^{fl/fl}$ wounds is most likely a result of elevated wound gelatinase expression. Moreover, the increased local collagen gene expression may reflect a compensatory mechanism. It is noteworthy that we have previously reported (Hardman *et al.*, 2005; Hardman and Ashcroft, 2008) a global compensatory upregulation of collagen gene expression in tandem with increased local gelatinase activity conserved across delayed-healing wounds in both humans and mice. Intriguingly, Arg1 is reportedly localized to gelatinase granules in human neutrophils (Jacobsen *et al.*, 2007). The provisional matrix laid down following healing is essential to provide a scaffold for the migration of key cell types and for wound-vessel

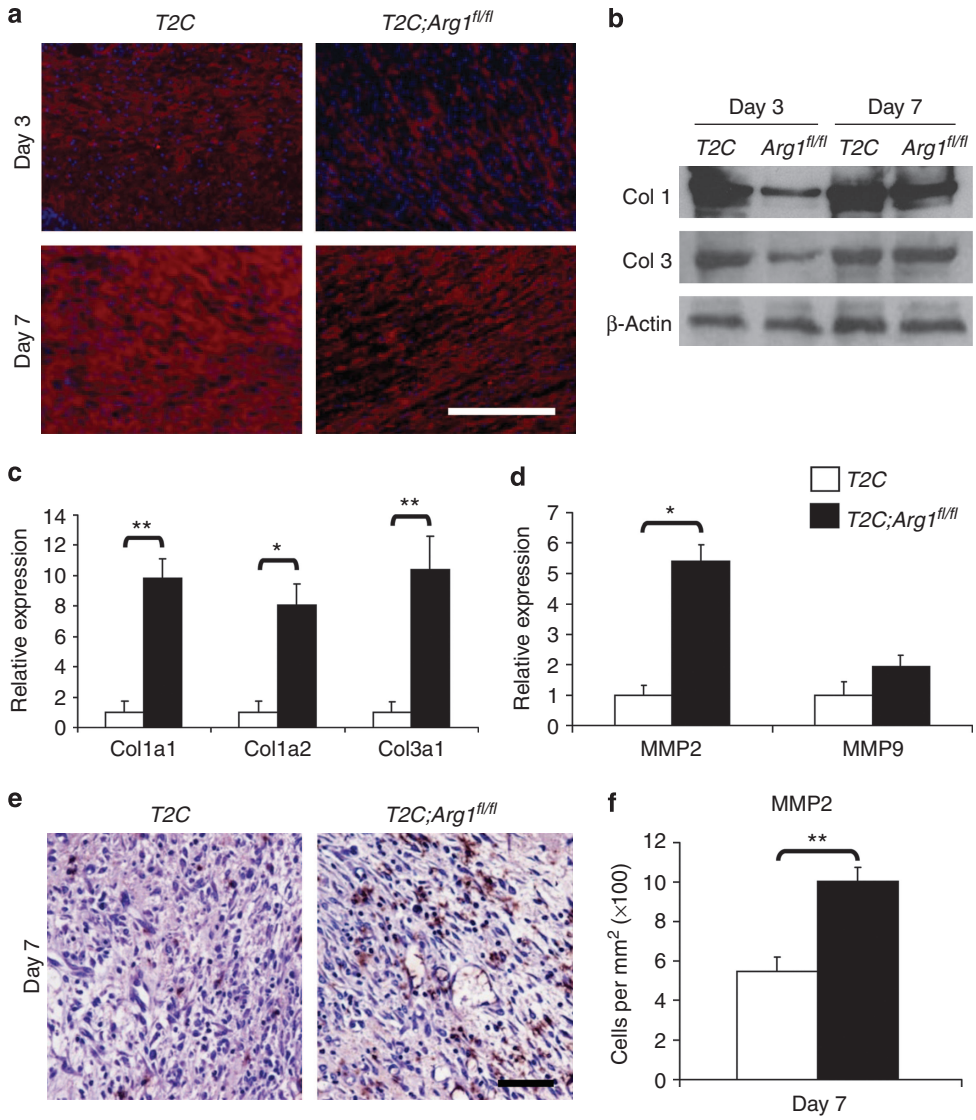

Figure 5. *T2C;Arg1*$^{fl/fl}$ wounds display altered matrix deposition and protease activity. Wound protein collagen 1 content determined by (**a**) immunofluorescence with corresponding western blot analysis for (**b**) collagen (Col) 1 and 3 revealed a reduction in *T2C;Arg1*$^{fl/fl}$ wounds. (**c**, **d**) *T2C;Arg1*$^{fl/fl}$ wounds display increased expression of collagen species and MMP2 (quantitative PCR) at 7 days post wounding. (**e**) Images representing the experimental group mean for MMP2^{+} dermal cells at 7 days post wounding. (**f**) Quantification of MMP2^{+} dermal cells reveals significantly increased numbers in *T2C;Arg1*$^{fl/fl}$ day 7 wounds. Immunohistochemical quantification data are derived from the mean of five randomly selected high-powered fields per wound and two wounds per mouse. Data presented indicate (**f**) mean + SEM of $n = 6$–7 mice per group, (**a**) $n = 4$ mice per group, or (**c**, **d**) three replicates per group and two individual experiments. Bar = 200 μm (**a**) and 50 μm (**e**). $^{**}P < 0.01$, $^{*}P < 0.05$.

neogenesis (Shaw and Martin, 2009), and matrix changes may thus be an important causative factor to the observed healing delay.

Altered macrophage polarization is emerging as a common theme across murine delayed-healing acute wound models: aged and ovariectomized mice (this study) and diabetic mice (Miao *et al.*, 2012). This fits with the hypothesis that delayed-healing wounds are unable to switch to a reparatory AAM environment required for normal healing. The finding that normal acute healing requires a temporal shift in macrophage polarization is supported by previous studies (Deonarine *et al.*, 2007) and our own data (Figure 1). A failure to switch from a Th1 to a Th2 environment would have severe consequences for the healing of chronic wounds. Here, we note confusion in the literature as to whether chronic wounds *per se* have altered Arg expression; an initial study showed increased Arg expression in diabetic ulcers versus normal skin (Jude *et al.*, 1999; Abd-El-Aleem *et al.*, 2000) supported by two subsequent reports of increased Arg expression in diabetic murine models (Kampfer *et al.*, 2003; Miao *et al.*, 2012). Importantly, these studies measured global wound Arg expression failing to account for potential variation in cellular source. Our data currently reveal that age-associated delay in acute healing is accompanied by a local reduction in wound granulation tissue Arg1^{+} cells.

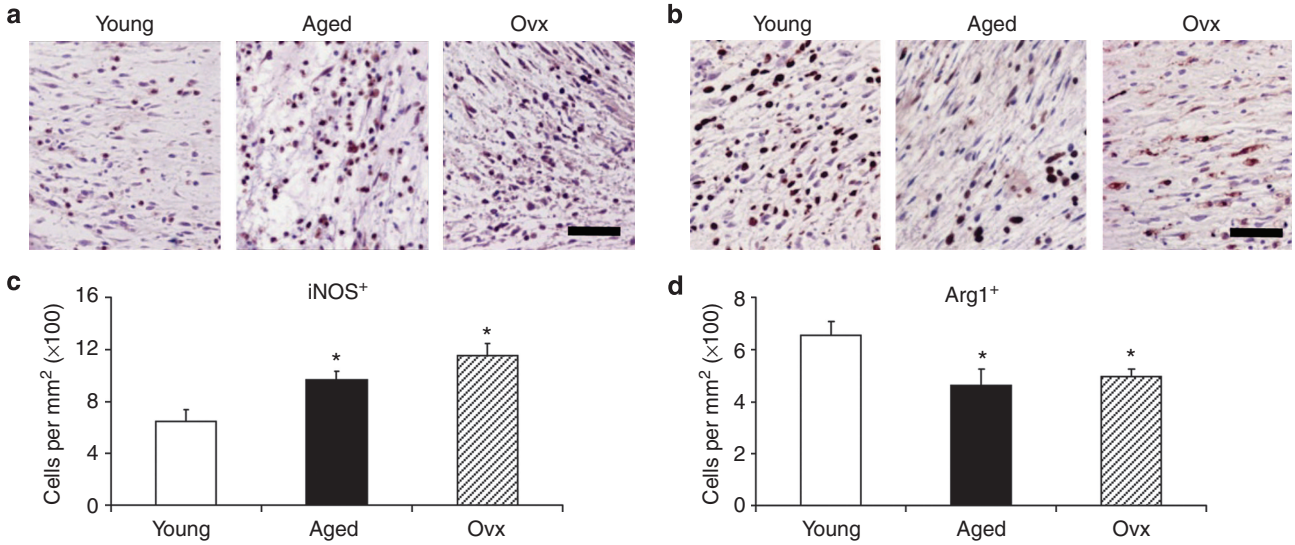

Figure 6. Reduced Arginase1 (Arg1) is a conserved feature of delayed healing in mouse and human wounds. Images representing the experimental group mean for (**a**) inducible nitric oxide synthase-positive (iNOS$^+$) and (**b**) Arg1-positive (Arg1$^+$) immunohistochemical analysis from control (young) and delayed healing aged and ovariectomized (Ovx) day-3 wounds. Aged and Ovx mice wounds are associated with increased numbers of (**c**) iNOS$^+$ dermal cells and (**d**) reduced Arg1$^+$ cells compared with control mice. Immunohistochemical quantification data are derived from the mean of five randomly selected high-powered fields per wound and two wounds per mouse. Data presented indicate the mean + SEM, $n = 6$ mice per group. Bar = 50 μm. *$P < 0.05$.

Data presented in this study clearly demonstrate an important and previously unappreciated role of Arg1 during cutaneous healing. These findings are particularly interesting in the context of previous studies demonstrating the beneficial effects of L-arginine supplementation on acute wound healing. An important next step will be to confirm potential beneficial effects of Arg1 and/or L-arginine in human chronic wounds. Indeed, we suggest that a combined L-arginine supplementation/local Arg induction therapeutic approach may have considerable clinical benefit.

MATERIALS AND METHODS

Animals and wounding

All animal studies were performed in accordance with Home Office regulations. Ten-week-old female C57BL/6, transgenic *Tie2cre;Arg$^{fl/fl}$* (El Kasmi *et al.*, 2008), *Tie2cre;Arg$^{+/+}$* (wild-type controls), ovariectomized (bilateral ovariectomy performed 3 weeks before wounding), and aged mice (18-month old) were anesthetized and wounded following our established protocol (Ashcroft *et al.*, 2003). N(omega)-hydroxy-nor-L-arginine (nor-NOHA—20 μg per 50 μl) (VWR International, Lutterworth, UK) or vehicle (phosphate-buffered saline) were locally injected in C57BL/6 mice at days 1 and 0 with respect to wounding and every subsequent day until collection. Two equidistant 1-cm full-thickness incisional or 6-mm excisional wounds were made and left to heal by secondary intention. Wounds were excised at either 1, 3, 5, 7, or 10 days post wounding and bisected, with half processed for histological analysis (wound midpoint). The remaining half of each wound was flash frozen and stored at −80 °C for biochemical analysis.

Histology and immunohistochemistry

Histological sections were prepared from wound tissue fixed in 10% buffered formalin saline and embedded in paraffin. Six-micrometer-thick sections were stained with hematoxylin and eosin or subjected to immunohistochemical analysis with the following antibodies: Arg-I goat polyclonal, NOS2 rabbit polyclonal and MMP2 goat polyclonal (Santa Cruz, Heidelberg, Germany), anti-neutrophil rat polyclonal (Fisher Scientific, Loughborough, UK), anti-Mac-3 rat polyclonal (BD Biosciences, Oxford, UK), Ym1 goat polyclonal (R&D Systems, Minneapolis, MN), and Collagen 1 rabbit polyclonal (Millipore, Billerica, MA). Bound primary antibody was detected using the VECTASTAIN ABC kit (Vector Laboratories, Peterborough, UK) combined with NovaRed substrate. Images were captured (Nikon eclipse E600/SPOT camera (Image solutions, Preston, UK)) and granulation tissue wound area and re-epithelialization quantified using Image Pro Plus software (MediaCybernetics, Rockville, MD) as previously described (Ashcroft and Mills, 2002). Total cell numbers (expressed as number of cells per mm^2) were determined using five randomly assigned granulation tissue images per wound with Image Pro Plus software.

Arg activity assay

Arg activity was assessed by measuring the amount of urea production via the metabolism of L-arginine by Arg as previously described (Corraliza *et al.*, 1994). In brief, wounded tissue was homogenized in 0.5 ml 0.1% Triton X-100 (Sigma-Aldrich, Cambridge, UK). After 30 minutes, 0.5 ml of assay buffer (10 mmol l^{-1} MnCl in 50 mmol l^{-1} Tris, pH 7.5) was added and the enzyme was activated by heating for 10 minutes at 55 °C. For the metabolism of L-arginine by Arg, triplicate cultures of 25 μl cell lysate in buffer were incubated with 25 μl of 0.5 M L-arginine (Sigma-Aldrich) for 60 minutes at 37 °C and the reaction was stopped by adding 400 μl of acid mixture. Twenty-five microliters of 9% α-isonitrosopropiophenone (Sigma-Aldrich) was added and incubated for 45 minutes at 100 °C in the dark. Absorbance was measured at 570 nm using a MRXII (Dynex Technologies, West Sussex, UK). To normalize the samples, the protein concentration in cell lysates was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Runcorn, UK).

Protein extraction and immunoblotting

Total protein was extracted from unwounded and wounded tissue by boiling and homogenizing in SDS sample buffer, containing 5% β-mercaptoethanol, for 5 minutes. Protein samples (1 mg) were separated by SDS–PAGE and blotted onto a nitrocellulose membrane, which was blocked in 5% nonfat milk and 0.1% Tween for 16 hours at 4 °C, before incubation with primary antibody for 1 hours at room temperature. The bound primary antibodies were detected with peroxidase-labeled secondary antibodies (GE Healthcare, Hatfield, UK), followed by the ECL Plus detection system (GE Healthcare). Primary antibodies against Arg I and Collagen 3A1 (Santa Cruz), Collagen I (Millipore), and β-actin (Sigma-Aldrich) were used in conjunction with anti-goat, anti-rabbit, or anti-mouse secondary antibodies (GE Healthcare).

Quantitative real-time PCR

Total RNA was isolated from frozen tissue by homogenizing in Trizol reagent (Invitrogen, Paisley, UK). cDNA was transcribed from 1 μg of RNA (Promega RT kit, Madison, WI and AMV-reverse transcriptase; Roche, Welwyn Garden City, UK) and quantitative PCR performed using the MESA-green kit (Eurogentec, Southampton, UK) and an Opticon quantitative PCR thermal cycler (Bio-Rad, Hemel Hempstead, UK). For each primer set, an optimal dilution was determined and melting curves were used to determine amplification specificity. Each sample was serially diluted over three orders of magnitude, and expression ratios were normalized to the mean of two separate reference primers (*Gapdh* and *Ywahz*) with all samples analyzed concurrently. Full primer sequences are listed in Supplementary Table S1 online.

Statistical analysis

Statistical differences were determined using either Student's *t*-tests (Figure 5) (Mann–Whitney *U* tests for nonparametric data), one-way analysis of variance (Figures 1c and 6) or two-way (Figures 1b, 2, 3, and 4) analysis of variance (with appropriate *post-hoc* testing) (SimFit, William Bardsley, University of Manchester, Manchester, UK). A *P*-value of <0.05 was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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