Enforced Expression of Hoxa3 Inhibits Classical and Promotes Alternative Activation of Macrophages In Vitro and In Vivo

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The regulated differentiation of macrophages (mços) and their subsequent activation into proinflammatory or prohealing subtypes is critical for efficient wound healing. Chronic wounds such as diabetic (db) ulcers are associated with dysregulation of macrophage function. Whereas non-db mços polarize to an M2-like, prohealing phenotype during the late stages of healing, db-derived mços continue to display an M1-like, proinflammatory, or a mixed M1-like/M2-like phenotype. We have previously shown that sustained expression of Hoxa3 reduces the excessive number of leukocytes within the db wound; however, the effect of Hoxa3 on mço polarization was unknown. In this study, we show that Hoxa3 protein transduction of mços in vitro enhances macrophage maturation, inhibits M1 polarization, and promotes M2 polarization, in part via regulation of Pu.1/Spp1 and Stat6. Sustained expression of Hoxa3 in vivo in db wounds reduces the number of Nos2+ (M1-like) mços, increases the number of Arg1+ and VEGF+ (M2-like) mços, and accelerates healing in a DNA-binding independent manner. Our findings suggest a role for Hox protein activity in promoting M1-to-M2-like phenotypic switching via interactions with myeloid transcription factors and provide insight into mechanisms regulating this process in db wound healing. The Journal of Immunology, 2016, 197: 872–884.

The number of patients suffering from chronic wounds is nearing epidemic proportions. Patients with both type 1 and type 2 diabetes have impaired wound healing, and 15% of these patients go on to develop chronic, nonhealing wounds, 84% of which result in lower limb amputation (reviewed in Ref. 1). Current treatments are not effective because of a poor understanding of the mechanisms underlying chronic wounds; thus, they are the leading cause of nontraumatic amputations in the developed world today (2, 3). During the past two decades, the role of dysregulated inflammation in impaired healing associated with aging and diabetes has become apparent. This is due in part to increased and prolonged expression of proinflammatory cytokines and chemokines, such as TNFα and IL-1β (4) as well as a decrease in growth factors such as fibroblast growth factors, TGF-β, platelet-derived growth factor, and vascular endothelial growth factor (VEGF), which normally function to promote new tissue growth and may also act as anti-inflammatory factors (5–8). However, therapies targeted at manipulating these factors alone are not sufficient to control chronic inflammation, and only a mild improvement in wound healing has been observed in the clinic. Therefore, a stronger focus on reprogramming cell behavior in the wound, in particular the therapeutic manipulation of macrophages (mços), could be an effective strategy.

mços are involved in all phases of wound repair and regeneration (9, 10). During the early stages of healing, proinflammatory (M1-like) mços dominate the wound environment, where they are involved in killing pathogens and removing debris. As the wound progresses to late stages of healing, most mços acquire the prohealing (M2-like) state that is critical for healing progression (11–13). In mice, the M2-like state is characterized by high levels of arginase production (11, 14). M2 mços can be generated in vitro by the stimulation of the IL-4R and/or IL-13R, which signal via the phosphorylation of Stat6 (15–17). This pathway is also important to wound healing in vivo (18). However, the transition of proinflammatory mços to prohealing mços is inhibited by pathological environments, such as aging (19) or diabetes. In addition, diabetes affects the process of how progenitor cells mature in the bone marrow (BM), how myeloid cells infiltrate the wound tissue, and how mços respond to signals from the environment (13, 20, 21). Mços from diabetic (db) mice continue to display these defects even when cultured outside the db environment, showing an aberrant polarization response to both pro- and anti-inflammatory stimuli (12, 13, 22–24). Extrinsic signals coming from local cells and Th2 lymphocytes, including IL-4, TGF-β, and IL-10 are insufficient to induce the phenotypic switch from M1-like to M2-like mços (13, 22). This deficit in reparative mços is associated with decreased angiogenesis, extracellular matrix remodeling and wound contraction (reviewed in Ref. 25). Therefore, there is a need to identify factors that can stimulate the phenotypic switch of mços within the wound from a proinflammatory to a prohealing phenotype to promote resolution of inflammation and wound healing.

Enforced expression of Hoxa3 has been shown to promote resolution of inflammation and wound healing in db wounds (21, 26). Hoxa3 is a member of the homeobox gene family which
encode master regulator transcription factors that specify segmental identity along the anterio-posterior axis (27). In addition, many Hox genes are expressed in early hematopoietic progenitors where they direct lineage commitment, cell proliferation, and self-renewal (28–30). Hox proteins can be delivered to cells directly via their capacity to cross-biological membranes in a receptor- and energy-independent manner (31, 32). Importantly, protein transduction of Hox proteins is a potentially safer method than gene transfer. For example, Hoxb4 protein transduction can promote hematopoietic stem cell self-renewal without inducing leukemia (33).

Enforced Hoxa3 expression in hematopoietic progenitors was shown to promote their differentiation into Gr1+CD11b+ cells, a proangiogenic myeloid population, and rescue their diabetes-induced defects (34). However, the specific effects of enforced Hoxa3 on mice were unknown.

We found that Hoxa3 protein transduction had an effect on both mature maturation and activation and attenuated the inflammatory phenotype of mice, possibly via regulation of Spi-1/Pu.1, an Ets family transcription factor that is required for the generation of mature mature mice (35–38). Our data show that Hoxa3 inhibits M1-like proinflammatory gene expression and induces expression of markers of M2-like/prohealing mice as well as increasing Stat6 phosphorylation, showing a link between Hoxa3 function and the IL-4 signaling pathway. Furthermore, sustained expression of Hoxa3 in vivo in db wounds also promotes the switch from an M1-like, proinflammatory phenotype to an M2-like, prohealing phenotype. These findings have therapeutic relevance because this switch in mouse phenotype is a key event in efficient wound healing (13, 24).

Materials and Methods

Animals

All animals were housed at the University of Manchester animal care facility, and all procedures were approved by the local ethical review committee and the Home Office and were performed in accordance with the requirements of the Animal (Scientific Procedures) Act 1986. Leprinb−/−, the most well-characterized mouse model of type 2 diabetes, and heterozygous control Leprinb+/- mice were purchased from Harlan (Oxfordshire, U.K.) or The Jackson Laboratory (Bar Harbor, ME). All animals used were 8–16 wk old and were age- and sex-matched to controls. A minimum of three biological replicates per group was used for in vitro experiments. For in vivo wound healing studies, six mice per group were used.

Wound model

Leprinb−/− (type 2 diabetes model) and heterozygous control Leprinb+/- (non-db) mice were anesthetized, and the dorsum was shaved and sterilized with penicillin/streptomycin, 300 mM NaCl, 1 mM BSA, 2% Igepal, 1 mM EDTA, 1% protease inhibitor mixture (Protenuz, and 1% sodium orthovanadate. Two micrograms of rabbit anti-m-Cherry (BioVision) was labeled with biotin (DSB-X); Molecular Probes) and incubated with 50 µg protein lysate, together with 0.4 µg prewashed Dynabeads (FlowComp Flexi system; Invitrogen). The mixture was left overnight at 4°C on a rotator before washing and elution with immunoprecipitation wash buffer (0.1% SDS, 500 mM Tris-acetate [pH 7.5], 300 mM NaCl, and 0.5% Igepal) using a magnet (EasySep; StemCell Technologies).

CM from 293T cells transfected with SP-Hoxa3mCherry or SP-m-Cherry and purified lysate from mice treated with CM were analyzed by Western blotting using 10% SDS-PAGE and polyvinylidine difluoride membrane, using the Bio-Rad Turbo Transfer System. Blots were probed at 4°C overnight with 0.1 µg µL-1 anti-m-Cherry Ab in a volume of 5 µL. One microgram of RNA was used for the reverse transcription reaction using BioScript RT (Bioline) and random primers in a total volume of 25 µL. The resulting cDNA was diluted to 1:15 to be used as a template for quantitative RT-qPCR (qRT-PCR).

Gene expression analysis

qRT-PCR analysis was performed using the following TaqMan Gene Expression Assays: Il17a (Mm00444551_m1), Il17b (Mm00445286_m1), Ifng (Mm00436652_m1), Ym11Chi313 (Mm00767889_m1), Arg1 (Mm00475988_m1), Tgfb (Mm01178820_m1), Mcl1 (Mm0132962_m1), Nos2 (Mm01308977_m1), Tnf (Mm00443258_m1), Ccl2 (Mm00444543_m1), C2 (Mm00444242_m1), Spi1 (Mm00448812_m1), Stat6 (Mm01160777_m1), and Socs1 (Mm00782580_s1). Hpyq61 (Mm00833431_g1) and Hotdog (Mm00501974_s1) were used as reference genes. Reactions were performed on a StepOnePlus cycler (Applied Biosystems) and analyzed using the 2-ΔΔCt method. All analyses were performed using Microsoft Excel 2013.

Communoprecipitation of Hoxa3 and Spi-1/Pu.1 protein

293T cells cotransfected with 5 µg pSport6-Pu.1 and pcDNA3.1-Hoxa3mCherry were harvested 48 h posttransfection. Cell lysates obtained using 1% Triton X-100 and 0.01% Igepal in PBS. Two micrograms of rabbit anti-m-Cherry (BioVision) and/or goat anti-Pu.1 (D-19; Santa Cruz Biotechnology) were labeled with biotin (DSB-X). Cells lysates (300 µg) were incubated with biotinylated anti-m-Cherry or anti-Pu.1 and then mixed with prewashed Dynabeads, according to the manufacturer’s instructions. The eluate was then analyzed by Western blot.
Luciferase reporter assay
Rat RAW 264.7 mefs (ATCC-LGC Standards) were treated with 20% M-CSF and 50% CM from either mCherry or Hoxa3mCh cultures for 24 h. Cells were transfected with 1 μg pMAX enhanced GFP (eGFP), 1 μg pSpOt-Pu.1, and 1 μg pGL-2.7/2μs luciferase vector (40) with a 1:4 ratio of FuGENE HD transfection reagent (Promega) and incubated for 24 h. Cell eGFP expression was measured by fluorescence microscopy, and luciferase activity assayed using the Lucifire Assay System (E1500), according to the manufacturer’s protocol (Promega). The level of luciferase activity is shown as relative light units calculated as light units/eGFP fluorescence.

mef activation
Hoxa3mCh- or mCherry-treated mefs from db or non-db mice were split into the following three groups: 1, nonactivated mefs; 2, mefs that were classically activated (CA) using serum-free mef medium supplemented with 100 ng/ml LPS (Sigma-Aldrich) and 100 ng/ml IFN-γ (Sigma-Aldrich); and 3, mefs alternatively activated (AA) using serum-free medium supplemented with 20 ng/ml IL-4 (PeproTech) and 50 μg/ml anti-IFN-γ. Twenty-four hours postactivation, RNA was harvested from each group independently for gene expression. Forty-eight hours postactivation, cells were harvested or supernatants collected for arginase assays, ELISA, and independently for gene expression. Forty-eight hours postactivation, cells were harvested or supernatants collected for arginase assays, ELISA, and Greiss assays.

NO release (Greiss assay)
NO release was determined using supernatants from mefs with Greiss reagents, as described previously (41). A colorimetric plate reader was used to measure absorbance at 570 nm and background absorbance at 630 nm.

Arginase activity
Arginase activity in mef lysates was measured using the spectrophotometric method, as described previously (42). Briefly, 10 mM MnCl2 and 0.5 M L-arginine were sequentially added to the mef lysates and incubated for 1 h at 37°C. The reaction was stopped by the addition of an acid solution, and the formation of urea by arginase was analyzed by adding isonitrosoisopropiophenone at 100°C for 45 min. The colored product was measured at 570 nm and the background absorbance at 450 nm.

ELISA
Detection of mouse TNF-α and IL-12 was determined using a cytokine-specific ELISA kit (eBioscience) as described previously (13). For mouse TGF-β, a Ready-Set-Go mouse TGF-β ELISA kit (eBioscience) was used according to the manufacturer’s instructions.

Wound processing for immunofluorescence staining
Wounds were processed from three type 2 db or control non-db mice treated with CMV-Hoxa3 or plasmid control as described in (19). Wounds were stained as described previously, with some modifications (13). In brief, 5 μm sections were generated from formalin-fixed wounds and stained with H&E using a Shandon Varistain 24-4 (Thermo Scientific) to locate the granulation tissue and peri-wound dermis. Matching sections were labeled with the following Abs diluted in PBS + 0.1% Tween 20: rat anti-Mac3 (1:100, clone M3/84 (RUO); BD Pharmingen), goat anti-Arg1 (1:200, sc-18354; Santa Cruz Biotechnology), rabbit anti-Nos2 (1:100, sc-651; Santa Cruz Biotechnology), rabbit anti-Vegf (1:400, EP1176Y; Abcam), and rabbit anti-Tgfβ (1:150, EPR12678(B); Abcam). Next, the following secondary Abs were applied: donkey anti-rat Alexa Fluor 488 (1:500, A-21208; Invitrogen Life Technologies), donkey anti-goat Cy5 (1:500, ab65666; Abcam), and donkey anti-rabbit Alexa Fluor 555 (1:500, A-31572; Invitrogen Life Technologies). Four fields from each wound were imaged for statistical analysis of Mac3+, Nos2+, Arg1−, and Mac3−Nos2+Arg1+. For VEGF and TGF-β staining, the number of Mac3+, VEGF+, and Mac3+VEGF+ or Mac3+, TGFβ+, and Mac3+TGFβ+ cells were determined. All sample analyses were performed using Imaris 3D/4D image processing and analysis software (Bitplane). The percentage of each phenotype was calculated as the mean ± SEM of all images in each category (n = 12).

Western blotting
Whole-cell lysates were obtained from mouse mefs at the specified time points by lysing the cells in complete radioimmunoprecipitation lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 2 mM sodium orthovanadate, and 1× protease inhibitor mixture [Fisher, BPE9706-1] in PBS). Phosphatase inhibitor was added to the lysis buffer when harvesting cell lysates for pStat6 detection. Cell lysate (10 μg) was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blot was incubated with either goat polyclonal anti-Pu.1 (1:200, D-19, sc-5949; Santa Cruz Biotechnology), rabbit monoclonal anti-Stat6 (1:2000, YE361, ab32520; Abcam), or rabbit polyclonal anti-pStat6 (1:1000, Tyr694, ab195647; Abcam). Protein was detected using ECL (Thermo Fisher Scientific).

Results
Protein transduction of Hoxa3 in mouse mefs promotes maturation
We and others have shown that mefs isolated from the db environment show differentiation and maturation defects, which likely contribute to their dysfunction in vivo (13, 22). Sustained expression of Hoxa3 in wounds of type 2 db mice results in significant reduction in the total number of inflammatory cells within the wound (21), but the mechanism underlying this effect on mefs was not identified. As protein transduction of Hoxa3 in hematopoietic stem cell/progenitors was shown to promote their differentiation into Gr1+CD11b+ cells, a prongiogenic granulocytic-monocytic cell population (34), we aimed to test whether overexpression of Hoxa3 by protein transduction on monocyte/mef precursors from type 2 db mice could rescue their differentiation defects. To test this, we used a construct in which Hoxa3 was fused to mCherry and to the Ig κ-chain signal peptide (Fig. 1A) to allow the secretion of Hoxa3 into the medium (43). Transfection efficiency of Hoxa3-mCherry (Hoxa3mCh) and mCherry control expression plasmids in 293T cells was monitored by live imaging (Fig. 1B). The presence of Hoxa3mCh and mCherry protein in the CM and in the targeted mouse mefs was confirmed via immunoprecipitation (Fig. 1C) and confocal imaging (Fig. 1D), respectively. Quantification of Hoxa3mCh and mCherry control protein localization within mefs was performed to validate appropriate nuclear localization of the Hoxa3mCh protein (Fig. 1E).

Following Hoxa3mCh or mCherry control protein transduction for 24 h, total RNA was isolated from target mefs and qRT-PCR conducted to measure expression of Emr1, encoding F4/80 (a mef-specific marker), Itgam, encoding CD11b (common myeloid marker), and Csf1r, encoding the M-CSF receptor (CD115) to assess mef maturation. Mefs derived from non-db and db mice showed significant increases in Emr1 (p < 0.05 and p = 0.07, respectively; Fig. 2A) and Csf1r (p = 0.05) in response to Hoxa3mCh. A significant increase in Itgam (p < 0.05) in the db-derived population was observed, but there was no apparent change in the db-derived mefs (Fig. 2A). We also analyzed changes in expression of these markers at the protein level by flow cytometry (Fig. 2B). Protein transduction of Hoxa3mCh showed a trend of increased mean fluorescence intensity (MFI) of cell surface F4/80 protein in non-db-derived mefs (Hoxa3mCh MFI = 27725.5 ± 13624 versus mCherry MFI = 17380.8 ± 13690, p = 0.2) and in the db-derived population (Hoxa3mCh MFI = 11746 ± 7681 versus mCherry MFI 8913 ± 5499, p < 0.1). CD115 was significantly increased by Hoxa3mCh protein transduction in non-db-derived mefs (Hoxa3mCh MFI = 10188.5 ± 2577 versus mCherry MFI 6213 ± 962, p = 0.05), and db-derived mefs (Hoxa3mCh MFI = 35915 ± 11729 versus mCherry MFI = 21824 ± 5971, p < 0.1). However, no detectable changes were noted in CD11b levels between Hoxa3mCh-treated and mCherry-treated mefs. Altogether, the Hoxa3-mediated regulation of mef maturation makers suggests that Hoxa3mCh protein transduction promotes the maturation of non-db- and db-derived mefs. The discrepancies in transcript and protein levels of Itgam may be due to posttranscriptional regulation of this protein, which has been shown in the db environment (13).
**Hoxa3 upregulates Spi1 levels in mouse mws**

Spi1 (also known as Pu.1), Cebpa, and Runx1 transcription factors are critical for monocyte and mws differentiation and are known to upregulate expression of myeloid differentiation markers, including Csf1r and Itgam (44). We hypothesized that Hoxa3 may promote maturation of mws via modulating the function of Spi1, Cebpa, and/or Runx1 transcription factors. We first assessed whether any differences were evident in the levels of expression of these transcription factors between non-db and type 2 db mws. The db-derived mws showed a significant reduction in Spi1 (**p**, 0.05; Supplemental Fig. 1A), whereas Cebpa and Runx1 levels did not show significant differences among the two populations (Supplemental Fig. 1B, 1C). We then assayed whether Hoxa3mCh protein transduction for 24 or 48 h could affect Spi1, Cebpa, or Runx1 mRNA levels. Interestingly, Hoxa3 upregulated Spi1 mRNA levels in non-db-derived mws after 48 h (**p** < 0.05; Fig. 3A) and in the db-derived mws at 24 h (**p** < 0.05) and 48 h (**p** < 0.005; Fig. 3A) but did not affect the levels of Cebpa or Runx1 (data not shown). We further investigated whether the changes observed in Spi1 mRNA levels were consistent with the Spi1/Pu.1 protein levels in non-db and db-derived mws. Surprisingly, Spi1/Pu.1 protein levels in non-db mws were significantly increased at 24 h following Hoxa3mCh treatment, despite a lack of significant increase in mRNA at this time point. However, as expected at 48 h following Hoxa3mCh treatment, we detected a ~14.8-fold increase in Spi1/Pu.1 protein (Fig. 3B). In the db-derived mws treated with Hoxa3mCh, Spi1/Pu.1 protein levels were also significantly increased by ~1.5-fold at 24 h and by ~7.4-fold at 48 h compared with control-treated cells (Fig. 3C). Levels of Spi1/Pu.1 were quantified relative to tubulin (Fig. 3D, 3E). These data suggest that Hoxa3 may induce Spi1/Pu.1 at the transcriptional and posttranscriptional levels. This Hoxa3-mediated upregulation of Spi1/Pu.1 protein in db-derived mws may be one mechanism by which Hoxa3 promotes mws maturation.

**Hoxa3 and Pu.1 proteins physically interact**

We next investigated whether Hoxa3 may physically interact with the Pu.1/Spi1 transcription factor and function as a cofactor on myeloid differentiation target genes. Other Hox proteins, such as Hoxc13, have been shown to interact with ETS proteins, including Pu.1/Spi1, by physical and functional interactions that are implicated in the differentiation of erythroid leukemia cell lines (27). We examined biochemical interactions between Pu.1 and Hoxa3 by performing immunoprecipitation assays on cell lysates from 293T cells that were cotransfected with mCherry-tagged Hoxa3 and Pu.1 constructs. The anti-Pu.1 Ab coimmunoprecipitated Hoxa3 but not the mCherry-only control (Fig. 3E), whereas the anti-mCherry Ab coimmunoprecipitated Pu.1/Spi1 in the Hoxa3mCh-Pu.1 cotransfection only (Fig. 3F), suggesting that Hoxa3 and Pu.1/Spi1 proteins physically interact.
To test whether this interaction affects Spi1 DNA binding or regulation of its target genes, we analyzed Spi1 activation of two Csf1r enhancers using a luciferase reporter assay system. Spi1 has been shown to physically bind to two enhancer regions near the Csf1r promoter, one upstream of the transcriptional start site and one downstream, in the first intron, and abolition of this binding results in loss of enhancer activity (45, 46). We found that protein transduction of Hoxa3 in combination with Spi1 significantly promoted Csf1r enhancer activity when both sites were present (pGL2-7.2fms construct) in response to M-CSF compared with control protein transduction (Fig. 3G). However, protein transduction of Hoxa3 did not affect the upstream-only reporter activation (pGL-0.5fms construct), suggesting it promotes Spi1 activation through the downstream enhancer (data not shown).

Hoxa3 inhibits M1 proinflammatory polarization of BMDMs

One of the reported functions of Hoxa3 in accelerated healing of the type 2 db mouse model is reducing the total number of inflammatory cells recruited to or retained in the wound via downregulation of NF-κB pathway genes (24). The NF-κB pathway promotes proinflammatory gene expression and an M1-like phenotype. On the basis of these results, we wished to test the ability of Hoxa3 to control the proinflammatory phenotype of mφs. Non–db- and type 2 db-derived mφs treated with Hoxa3mCh or mCherry CM were either left in a naive state (nonactivated [NA]) or CA using LPS and IFN-γ. Production of NO as well as the proinflammatory cytokines TNF and IL-12 were then assessed. We found that Hoxa3mCh significantly reduced NO production from CA mφs (Fig. 4A). In addition, the level of IL-12 cytokine production was reduced in CA Hoxa3mCh-treated non–db-derived mφs by more than 4-fold compared with control-treated cells (Fig. 4B). In db-derived mφs, Hoxa3 also inhibited IL-12 production in CA mφs by ~2-fold compared with control-treated cells (Fig. 4B). In a similar manner, Hoxa3 markedly reduced TNF production by ~53-fold in CA non–db-derived mφs (Fig. 4C) and showed a trend in reducing TNF production in CA db-derived mφs compared with controls (p = 0.18; Fig. 4D).

To determine whether protein transduction of Hoxa3 can limit the expression of M1 marker genes in CA mφs, we assessed the expression of M1 marker genes in NA and CA db and non–db-derived mφs (Fig. 4E–H). Hoxa3mCh-treated CA mφs showed significantly reduced expression of all M1 marker genes tested including NO synthase (Nos2; Fig. 4E), TNf (Fig. 4F), chemokine C-C motif ligand 2 (Ccl2) (Fig. 4G), and Cdd86 (Fig. 4H). These data, therefore, support the hypothesis that enforced expression of Hoxa3 can limit M1 mφ polarization in the presence of proinflammatory stimuli such as LPS and IFN-γ.
Hoxa3 promotes M2 prohealing polarization of BMDMs

Prohealing mços (M2-like) are the major source of growth factors such as TGF-β that contribute to tissue repair and wound healing (8). Previous work from our laboratory has shown that enforced expression of Hoxa3 upregulates Tgfb in myeloid cells (34), suggesting it may promote M2-like polarization. Therefore, we assessed the profile of Hoxa3mCh-treated mços for M2 markers such as arginase and TGF-β production. Arginase, a hallmark of AA mços in mice, was assessed by measuring urea in cell lysates, which is a product from arginase degradation of L-arginine. This assay showed significantly higher levels of urea in AA non-db-derived mços and a trend of increased urea levels in AA db-derived mços treated with Hoxa3mCh compared with control-treated mços (Fig. 5A). Hoxa3 also increased the levels of anti-inflammatory cytokine/growth factor TGF-β by ≈5-fold in AA non-db-derived mços (Fig. 5B) and by ≈2.9-fold in AA db-derived mços (p = 0.06; Fig. 5B). Further analyses of the regulatory effect of Hoxa3 protein transduction on levels of these M2 markers were obtained by performing qRT-PCR on Arg1, Mrc1 (mannose receptor CD206), Tgfb, and chitinase 3-like 3 (Chi3l3, also known as Ym1) on RNA
extracted from NA and AA mws treated with Hoxa3mCh or mCherry control CM. Hoxa3mCh-treated AA non–db- and db-derived mws showed significant upregulation of Tgfb, Mrc1, and Chi3l3 mRNA in AA non-db- and db-derived mws (Fig. 5C–F). Altogether, the data suggest that Hoxa3 promotes M2 polarization by reducing M1 markers and upregulating M2 markers, which may contribute to its capacity to promote wound resolution in vivo.

**Hoxa3 inhibits M1-like and promotes M2-like polarization of macrophages in day 7 db wounds**

The data above suggested enforced expression of Hoxa3 suppress the M1 phenotype and promote the M2 phenotype in cultured mws. We therefore wished to investigate whether treating type 2 db wounds with Hoxa3 could influence mws phenotype in vivo. Previous studies from our laboratory found that wounds of db mice show significant increases in M1-like (Nos2+) cells and significant decreases in M2-like (Arg1+) cells when compared with non-db wounds at day 7 following wounding (13). Because day 7 in the healing process represents the phase where most mws in non-db wounds have transitioned from a proinflammatory phenotype to a prohealing phenotype, we therefore investigated the effect of treating db mouse wounds with Hoxa3 at this time point. Sections were taken from full thickness excisional wounds that were treated with Hoxa3 or control and subsequently labeled with Abs for detecting Mac3 (pan-macrophage marker), Nos2 (M1 marker), and Arg1 (M2 marker). Analysis was performed on granulation...
tissue and peri-wound dermis (Fig. 6A). In Hoxa3-treated wounds, 7 d postinjury, triple staining for Mac3, Nos2, and Arg1 showed a trend of reduced Nos2+Arg1+ (M1-like) mws in the Hoxa3mCh-treated wound as compared with control treated and significantly increased numbers of Nos2-Arg1+ (M2-like) mws (Fig. 6B, 6D). There appeared to be no difference in the number of mixed phenotype Nos2+Arg1+ mws.

Additional markers for proreparative mws, TGF-β and VEGF, were also analyzed, as several previous studies have shown that TGFβ+ and VEGF+ macrophages are critical for effective healing (12, 47). Sections from db wounds treated with Hoxa3 or control were double labeled with Abs detecting Mac3 (mw marker) and VEGF or TGF-β, and analysis was performed as above. In Hoxa3mCh-treated wound as compared with control treated and significantly increased numbers of Nos2-Arg1+ (M2-like) mws (Fig. 6B, 6D). There appeared to be no difference in the number of mixed phenotype Nos2+Arg1+ mws.

Hoxa3 upregulates IL-4 signaling pathway effector Stat6 and increases phospho-Stat6 levels

The IL-4 signaling pathway strongly promotes M2 macrophage polarization via Stat6 activation. Upon IL-4 stimulation, Stat6 is phosphorylated by the activated JAKs, and translocates through the nucleus to function as a transcription factor that upregulates Arg1, Tgfβ1, Mrc1, and Chi3l3/Ym1 (M2 genes) (15, 16). To determine whether Hoxa3 promotes the M2-like mw phenotype by interacting with the IL-4 signaling pathway, we investigated Stat6 activation following Hoxa3mCh protein transduction in vitro. We assessed the levels of Stat6 transcript, and total Stat6 protein as well phospho-Stat6 in Hoxa3mCh-treated mws. We found that Hoxa3 upregulated Stat6 transcript and Stat6 protein levels in both non-db and db-derived mws following IL-4 stimulation (Fig. 7A–C). Interestingly Hoxa3 increased total Stat6 protein accumulation in the unstimulated cells (Fig 7C), although no detectable transcript difference was apparent (Fig. 7A, 7B), suggesting that Hoxa3 may also mediate stabilization of Stat6 protein. In addition, phospho-Stat6 was also markedly increased in the presence of Hoxa3 in non-db-derived and db-derived mws (Fig. 7C). Analysis of Hoxa3-treated whole wound samples from type 2 db mice also revealed up-regulation in Stat6 mRNA levels (Fig. 7D), suggesting Stat6 is a downstream target of Hoxa3 in vivo as well. We did not observe

![FIGURE 5. Analysis of prohealing (M2) mw markers in Hoxa3mCh- or mCherry-treated BMDMs from non-db (ndb) and db mice. (A) Arginase assay (urea production from L-arginine) in NA or AA mws from ndb or db mice treated with mCherry (N) or with Hoxa3mCh (n). (B) TGF-β production from NA or AA mws from ndb or db mice treated with mCherry (N) or Hoxa3mCh (n). (C-F) Relative expression (RE) of Arg1, Tgfb1, Mrc1, and Chi3l3/Ym1 (M2 genes) to Hsp90 (reference gene, n = 5; *p < 0.05, ***p < 0.005).]
any change in Il4 or Tgfb1 mRNA expression in vivo (data not shown), suggesting that Hoxa3 modulation of Stat6 is independent of these genes. We also analyzed Socs1 expression in response to Hoxa3, as Socs1 is known to be activated in response to IL-4 signaling, and is crucial for IL-4–dependent M2 polarization in mφs (48). Protein transduction of Hoxa3mCh significantly increased Socs1 expression in db-derived mφs and showed a trend of increased expression in non–db-derived mφs (Fig. 7E). Taken together, our findings suggest that enforced expression of Hoxa3 can promote alternative activation of mφs via increasing cellular pools of Stat6 and promoting Stat6 phosphorylation in murine mφs.

**Hoxa3 accelerates wound healing in type 2 db mice in a DNA-binding independent manner**

We have previously shown that enforced expression of Hoxa3 in db wounds accelerates wound healing (26). However, our prior work was performed using a larger excisional wound model than the current study. In addition, we wished to test whether DNA binding is required for this function. To do this, we induced a mutation to the reading frame encoding the homeodomain of Hoxa3 to change asparagine 51 to glutamine (N51Q), abolishing DNA binding (49). Interestingly, we found that the N51Q mutant was able to promote wound healing in db mice as effectively as the wild type Hoxa3 (Fig. 7F). This finding is consistent with a model in which Hoxa3 promotes macrophage maturation and polarization via interaction with other DNA-binding partners, such as Spi1/Pu.1.

**Discussion**

To assess the therapeutic potential of Hoxa3 on dysregulated mφs, we used an ex vivo culture model of BMDMs from type 2 db and non-db mice. Hox proteins possess the ability to translocate across biological membranes in an energy-free, endocytosis-free manner because of the penetratin peptide located in the third α helix of homeodomain proteins (31, 32). Using this property, we delivered...
Hoxa3 into cultured m\(\delta\)s using protein transduction. This method is potentially a safe and useful method to overexpress transcription factors ex vivo to reprogram cells for therapeutic use because it does not involve gene manipulation or possible damage to the cell that may be caused by other methods such as viral transduction. The passage translocation of Hox proteins was previously described with Hoxa3 and Hoxb4 using a coculture system to deliver them into hemopoietic stem cells (33, 34). In this study, we demonstrate for the first time, to our knowledge, the successful delivery of Hoxa3 protein into m\(\delta\)s using a CM enriched with Hoxa3 rather than the more complicated coculture system approach. This is superior to a coculture system as it is a step closer to good manufacturing practice conditions, which would be required for potential therapeutic use.

m\(\delta\)s are critical to wound healing as they secrete growth factors and cytokines necessary for wound resident cell proliferation, migration, and other behaviors (12, 50, 51). They also contribute to resolution of the inflammatory phase via efferocytosis (removal of dead/dying cells in the wound), particularly of neutrophils. The presence of neutrophils until late stages of healing in db wounds can negatively impact the healing process by causing oxidative damage and exacerbating tissue damage by secreting or leaking excessive proteases and elastases (52, 53). Khan et al. (54) found that successful efferocytosis can switch the m\(\delta\) phenotype in the db wound from proinflammatory to anti-inflammatory. Furthermore, several studies have suggested that db-derived m\(\delta\)s fail to perform this function effectively (55–57). However, until now it has not been clear whether delayed/failed maturation of db m\(\delta\)s

FIGURE 7. Hoxa3 regulation of IL-4-Stat6/phospho-Stat6 signaling pathway during M2 activation. Relative expression (RE) of Stat6 in mCherry (\(\oplus\)) or Hoxa3\(\text{mCh}\)-treated (\(\bullet\)) non-db (n\(\delta\)) and db-derived m\(\delta\)s (B), stimulated with IL-4, normalized to m\(\delta\)s without IL-4 stimulation (no IL-4). Hist2h2aa1 and Hsp90ab1 were used as reference genes, and values represent mean ± SEM of \(n=5\) biological replicates. *\(p<0.05\). (C) Representative Western blot of total protein lysates from m\(\delta\)s pretreated with Hoxa3\(\text{mCh}\) or mCherry CM that were left unstimulated (no IL-4) or stimulated with IL-4 and probed with the indicated Abs. Blots were repeated three times on m\(\delta\)s from three different mice. (D) Stat6 expression in day 4 wounds of db mice treated with empty vector (\(\oplus\)) or Hoxa3 expression plasmid (\(\bullet\)) relative to reference. (E) Socs1 expression in NA and AA n\(\delta\) non-db-derived m\(\delta\)s (upper graph) and db-derived m\(\delta\)s (lower graph) treated with control (\(\oplus\)) or Hoxa3\(\text{mCh}\) (\(\bullet\)). (F) Mean percent open wound following 0.8-cm excisional wounding on days indicated in n\(\delta\) mice treated with control plasmid (blue line), db mice treated with control plasmid (orange line), db mice treated with wild-type Hoxa3 plasmid (green line), and db mice treated with mutant Hoxa3 plasmid encoding a change in homeodomain aa 51 from Asn to Gln (N51Q). Hoxa3, \(n=6\) group, error bars indicate SEM, *\(p<0.05\) when comparing Hoxa3-treated db versus control-treated db. cntl, control.
may underlie defective efferocytosis in db wounds, leading to excessive accumulation of leukocytes. The link between abnormal maturation of myeloid cells and diabetes was demonstrated in non-obese db mice in which administration of GM-CSF promoted the maturation of DCs and subsequently reduced the development of diabetes (58) as well as in obese db mice in which administration of G-CSF–promoted Gr-1+CD11b+ cell maturation and wound healing (59); thus, the cause and effect relationship between these two states may be intertwined. Our data suggest that this relationship can be extrapolated to macrophages in diabetic chronic wounds as well.

Several studies have demonstrated that diabetes induces a dysfunctional macrophage polarization phenotype (13, 22, 60), even when cells are removed from the db environment and cultured ex vivo (13). Although environmental stimuli play a large role in macrophage phenotype, this last observation suggests a role for cell intrinsic factors in regulating macrophage maturity and phenotype as well. Transcription factors are likely candidates for such cell intrinsic factors because they control cell differentiation and phenotype by regulating hundreds of target genes that affect multiple processes within the cell. Hoxa3 has been shown to be aberrantly regulated in db wounds, and enforced expression of this potent transcription factor can significantly inhibit inflammation, promote angiogenesis, and accelerate healing (21, 26, 34). In this study, we investigated the effect of Hoxa3 protein transduction and in vivo gene transfer on macrophage maturation and phenotype both ex vivo and in vivo. Hoxa3-treated macrophages showed upregulation of maturation markers F4/80 and CD115 as well as a master regulator of macrophage development, Spi1/Pu.1, at both transcriptional and protein levels, thus supporting the hypothesis that Hoxa3 rescues diabetes-induced macrophage maturation defects. In addition, we found that Hoxa3 can directly interact with Spi1, suggesting Hoxa3 may act as a cofactor enhancing Spi1 function when overexpressed. Upregulation of Csf1r, a known downstream target of Spi1, supports this hypothesis. In addition, luciferase assays indicate Hoxa3 collaborates with Spi1 to activate the promoter/enhancer elements controlling Csf1r expression. However, transcription of Ilgam, which encodes CD11b and is another known target of Spi1 (61), was not upregulated by Hoxa3 overexpression, suggesting only a subset of Spi1 targets are modulated by Hoxa3 interactions. Interestingly, abolishment of Hoxa3 DNA-binding did not affect its ability to promote wound healing, providing further evidence that Hoxa3 modulates macrophage behavior via direct interactions with myeloid transcription factors. Further studies to clarify the importance of Hoxa3-Spi1 interactions on macrophage maturation and polarization during wound healing will address this question.

In addition to promoting macrophage maturation, Hoxa3 attenuated the proinflammatory response of macrophages to LPS and IFN-γ and reduced NO, IL-12, and TNF release in cell supernatants. Analysis of M1 gene expression supported the hypothesis that Hoxa3 inhibited M1 polarization. This is consistent with the previously described anti-inflammatory effect of Hoxa3 in vivo, where sustained expression of Hoxa3 reduced the recruitment and/or retention of inflammatory cells and resulted in downregulation of genes involved in multiple inflammatory pathways, including NF-κB (21).

In addition to suppressing the M1 polarization phenotype, enforced expression of Hoxa3 also promotes the M2 polarization phenotype. We found Hoxa3 upregulated Tgfb, a marker of M2 macrophages, at the mRNA level and increased TGF-β growth factor release from non–db- as well db-derived macrophages. This is consistent with a previous study showing upregulation of Tgfb in Gr-1+CD11b+ myeloid cells transfected with Hoxa3 (34). TGF-β functions during the later stages of wound healing to promote angiogenesis, stimulate myofibroblast differentiation.

**FIGURE 8.** Model of multiple effects of Hoxa3 on db macrophages. db-derived macrophages have aberrant maturation and dysregulated polarization phenotypes. Enforced expression of Hoxa3 in ex vivo–differentiated mouse macrophages promotes differentiation of macrophages and increases expression of F4/80 and CD115 via upregulation of the Spi1/Pu.1 transcription factor. Hoxa3 also rescues the aberrant polarization phenotype in db macrophages, promoting the M2/prohealing polarization state in BM-derived as well as in day 7 db wound macrophages. This occurs in part by Hoxa3–mediated increases in cellular pools of Stat6 protein as well as phosphorylation. In parallel, Hoxa3 also inhibits the heightened M1/proinflammatory macrophage phenotype in db macrophages, possibly by inhibiting NF-κB activation.
and wound closure, and promote collagen deposition (reviewed in Ref. 62). Hoxa3 treatment of wounds also increased the number of TGF-β-positive cells in the wound, although not specifically in macrophages. Hoxa3 treatment also increased arginase production in mice from both non-db and type 2 db mice. Arginase is thought to promote wound healing by shifting the production of NO to urea and ornithine. Ornithine is a precursor of polyamines and collagen (reviewed in Ref. 63).

Although arginase is not highly expressed in human monocytes or mice when stimulated in vitro (64), it has been shown to be expressed in human neutrophils (65), which may indicate that these cells play a similar role in human wounds with regard to arginase function. Stat6 function in murine and human cells is conserved, and thus, our finding that Hoxa3 treatment induces upregulation of Stat6 and increased phosphorylation of Stat6 will be relevant to human macrophage biology. Stat6-dependent gene regulation is critical to M2 macrophage behavior (reviewed in Ref. 11).

Impaired wound healing in both type 1 and type 2 db mouse models and human patients has been linked to a failure in mice to switch from the M1 phenotype to the M2 phenotype (13, 22, 60). In this study, we have shown that enforced expression of Hoxa3 in type 2 db-derived mice can promote this switch during wound healing via multiple mechanisms (Fig. 8). Hoxa3 treatment promotes mice maturation via upregulation of and direct interaction with Spi1, which may lead to enhanced M2 function. This in turn may attenuate proinflammatory responses and augment anti-inflammatory behaviors. Hoxa3 also inhibits M1 polarization via suppression of proinflammatory gene expression and promotes M2 polarization by enhancing IL-4 signaling through increased Stat6 activation. Altogether, these functions of Hoxa3 shift the balance from an M1-dominant wound environment to an M2-dominant wound environment, thus accelerating healing.

We conclude that driving intrinsic changes in M2s are key to overcoming aberrant environmental signals, which are present in tissues displaying chronic inflammation. Using protein transduction may be a potential therapeutic technology to safely reprogram inflammatory cell behavior in vivo. Future studies will be focused on addressing mechanisms underlying Hoxa3-Spi1 interactions and how regulation of M2 maturation may be involved in the M1-M2 transition.

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Disclosures

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