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Topical Fluoxetine as a Novel Therapeutic That Improves Wound Healing in Diabetic Mice

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Diabetic foot ulcers represent a significant source of morbidity in the U.S., with rapidly escalating costs to the health care system. Multiple pathophysiological disturbances converge to result in delayed epithelialization and persistent inflammation. Serotonin (5-hydroxytryptamine [5-HT]) and the selective serotonin reuptake inhibitor fluoxetine (FLX) have both been shown to have immunomodulatory effects. Here we extend their utility as a therapeutic alternative for nonhealing diabetic wounds by demonstrating their ability to interact with multiple pathways involved in wound healing. We show that topically applied FLX improves cutaneous wound healing in vivo. Mechanistically, we demonstrate that FLX not only increases keratinocyte migration but also shifts the local immune milieu toward a less inflammatory phenotype in vivo without altering behavior. By targeting the serotonin pathway in wound healing, we demonstrate the potential of repurposing FLX as a safe topical for the challenging clinical problem of diabetic wounds.

Chronic wounds represent a significant source of morbidity, with more than 6 million people suffering in the U.S. alone and expenditures of ~$9.7 billion annually (1). With standard of care, only 50% of patients with diabetic foot ulcers heal, and to date, no single therapeutic agent has been successful in improving the healing rate above 50–60% (2). During the wound healing process, the initial postwounding inflammatory phase, influx of neutrophils and macrophages, is critical for normal healing but, if persistent, results in a chronically inflamed wound that does not heal (1).

Our early finding of high levels of serotonin (5-hydroxytryptamine [5-HT]) generated by cultured human bone marrow mesenchymal stem cells (3), cells important for tissue repair, prompted this investigation into the utility of serotonin, or selective serotonin reuptake inhibitors (SSRIs) that increase extracellular serotonin, to improve wound healing. Serotonin receptors are widely expressed on many tissues, including cells present within the skin (4). A clinical trial of the effects of 5-HT in wounds demonstrated that ketanserin (a 5-HT receptor 2A [HTR2A] inhibitor) had no effects on healing of the normal surgical wound (5); however, it did improve healing in patients with venous or ischemic ulcers (6). Unfortunately, the study did not conform to current Consolidated Standards of Reporting Trials (CONSORT) guidelines for randomization method, exclusion of rapid healers, size limitations, and mixed wound etiologies, so conclusions are limited (5,6). Ketanserin may improve wound healing by blocking the vasoconstrictive effects of 5-HT on HTR2A (6). Intriguingly, we found that 5-HT itself appears to be beneficial in an in vivo animal model of impaired healing. We set out to determine additional targets of serotonin signaling that may mediate the observed improvement in healing.

Fluoxetine (FLX) is an SSRI that is used to treat psychiatric disorders. Interestingly, prior studies have shown that FLX has immune-modulatory properties (7).

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For example, lymphocytes are activated in patients with depression with dysfunctional serotonergic systems, and FLX administration reduces their proliferation and immune function (8). Therefore, examining SSRI local effects on the stalled inflammatory phase that characterizes chronic wounds is reasonable.

Here we demonstrate the utility of repurposing FLX as a topically applied drug to improve healing, with action on multiple targets of wound healing, including improved re-epithelialization and decreased inflammation.

RESEARCH DESIGN AND METHODS

Protocols Approved

Both institutional review board and institutional animal care and use committee approval were obtained for all human tissue and animal experiments.

Primary Neonatal Human Keratinocyte Isolation and Scratch Wound Assays

Neonatal human keratinocytes (NHKs) were isolated from human foreskin, and assays were performed as previously reported (9).

Time-lapse images of wounded cultures were captured every 30 min for 6 h. Healing was calculated as follows:

$$\%\ \text{healed} = \frac{SA_{t=0} - SA_{t=6}}{SA_{t=0}}$$

SA represents surface area of the scratch wound gap at the 0-h (t = 0) or 6-h time point.

In Vivo Wounding

Mice were randomly assigned to control or treatment groups to limit bias. Diabetic (*db/db*) mice (age 11 weeks, blood glucose >300 mg/dL) received two full-thickness 8-mm splinted circular excisional wounds as previously described (10). Daily treatments as indicated were applied topically. Day 10 wound tissue was fixed, sectioned, and stained for hematoxylin-eosin or immunohistochemistry.

To limit potential bias in scoring results of scratch wound assays and histological evaluation of wound re-epithelialization, images were captured, coded, and scored by investigators blinded to the treatment group. To limit potential bias in the measurement of wound re-epithelialization, wounds were bisected through the center of the lesion and multiple sections obtained in order to score the section with the largest wound diameter. In a prior publication (10), we reported on the experimental conditions that can affect outcomes in interpretation of healing of splinted wounds in mice and have incorporated the optimized conditions in the work reported here.

Flow Cytometry

Wound tissues were dissociated mechanically (Tissue Tearor; BioSpec Products) and enzymatically (Dispase II, Collagenase D, DNase I; Sigma-Aldrich). Flow was performed with the following monoclonal antibodies: peridinin chlorophyll protein complex (PerCP)–conjugated anti-CD45 (30-F11), BV711-conjugated anti-CD11b (M1/70), phycoerythrin-conjugated anti-Ly6C (Hk1.4), FITC-conjugated anti-Ly6G (1A8), phycoerythrin-Cy7–conjugated anti-CD11c (N418), APC-conjugated anti-F4/80 (BM8), APC-Fire–conjugated anti-MHC II (M5/114.15.2), and BV421-conjugated anti-CD206 (C068C2) (all from BioLegend, San Diego, CA). Data were acquired using an Attune NxT Flow Cytometer (Invitrogen by Thermo Fisher Scientific) and were analyzed using FlowJo software (Tree Star Inc.).

Multiplex Assays

Wound lysates were assayed according to the manufacturer's protocol (Millipore Multiplex Assay; MILLIPLEX MAP 48-680MAG).

RT-PCR

RNA was extracted from wound tissue using Qiazol (Qiagen) followed by RNeasy Miniprep (Qiagen). RNA was reverse transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad). RT-PCR was performed using PowerUp SYBR Green Master Mix (Thermo/Applied Biosystems). Data were analyzed and normalized using the ΔΔCt method with GAPDH, Tbp, and 18S rRNA as housekeeping genes. Fold change is shown relative to the healthy unwounded skin.

Statistical Analysis

Kolmogorov-Smirnov tests were performed to verify normal distribution of each data set. For data that do not deviate from normal distribution, Student t test was used to compare each individual treatment group to the control; ANOVA was used to determine statistical significance when there were three or more groups of treatment. For data that did not pass the normality test, the nonparametric Mann-Whitney test was used to assess statistical significance. P values ≤0.05 were considered significant.

RESULTS

Wound re-epithelialization, needed for complete healing, requires migration of keratinocytes from the wound edge, a function that is stalled in chronic wounds (11). Using an in vitro scratch assay to evaluate keratinocyte migration and ability to re-epithelialize a wound, we found that serotonin (5-HT) enhanced migration in a dose-dependent manner (Fig. 1*A*). Control untreated cultures healed 41.5% of their scratch wound area compared with 52.2% in the 1 μmol/L 5-HT treatment group (P = 0.01) and 54.7% in the 10 μmol/L 5-HT treatment group (P = 0.001) (Fig. 1*A*). To explore the signaling that modulated migration in these cells, we probed relevant intracellular signaling pathways using protein multiplex assays. We discovered that 5-HT activated mitogen-activated protein kinase (MAPK) pathways in keratinocytes, evidenced by the increase in phosphorylation of ERK (1.34-fold, P = 0.004) and its downstream signals STAT3 (1.59-fold, P = 0.043) and NF-κB (1.66-fold, P = 0.035). There was no modulation

observed in PI3K/Akt pathways (Fig. 1*B*). Although human keratinocytes have been shown to express tryptophan hydroxylase gene (12), an enzyme in the rate-limiting step in 5-HT synthesis, we did not find any 5-HT produced by keratinocytes above our lower limit of detection of 9.8 nmol/L (Supplementary Fig. 1). Thus, endogenous generation of 5-HT by keratinocytes may be too low to enhance migratory speed in FLX-treated keratinocytes in the absence of exogenous 5-HT (Fig. 1*C*). In the presence of 5-HT, however, FLX improved healing in treated cultures: 60.6% in the 10 nmol/L treatment group, 62.0% in the 100 nmol/L treatment group ($P = 0.01$), and 67.0% healed wound area in the 1 μmol/L FLX group ($P = 0.001$), relative to the 52.2% healing in the control cultures (Fig. 1*D*). FLX-enhanced keratinocyte migration effect was consistent with what we found in keratinocytes derived from patients with type 2 diabetes (Supplementary Fig. 1). These data support the hypothesis that signaling through the serotonin pathway increases keratinocyte migration in vitro. To provide further evidence that FLX is working through 5-HT–dependent pathways, scratch wound assays were repeated in the presence of the HTR2A blocker ketanserin. The HTR2A blocker ketanserin reversed the effects of FLX on wound healing in vitro, again returning wound healing to the level of the untreated control group ($P = 0.948$). These data demonstrate not only that FLX increases keratinocyte migration in vitro but that this is dependent upon 5-HT signaling through HTR (Fig. 1*D*).

To test whether FLX promotes re-epithelialization in vivo, full-thickness excisional wounds in *db/db* diabetic mice, a model for impaired wound healing (10), were treated with topically applied FLX, serotonin, or vehicle control 5% w/v polyethylene glycol (PEG). Wounds from mice treated with either 0.02% FLX or 2% 5-HT dissolved in 5% PEG showed decreased wound area and less exudate compared with vehicle control counterparts at day 10 postwounding (Fig. 2*A*). Moreover, re-epithelialization was increased from an average of 39.6% in PEG-treated mice to 66.2% in mice treated with 0.2% FLX ($P = 0.01$) (Fig. 2*B* and *C*). Since the topical application of 5-HT did not result in statistically significant improvement in re-epithelialization, likely due to the short half-life of serotonin (13), we did not further investigate its direct effects.

Immunohistochemical analysis showed increased $CD31^+$ endothelial cells in the wound beds of mice treated topically with 0.2% FLX (133 cells/mm^2), with more visible scattered small blood vessels compared with control mice (67 cells/mm^2, $P = 0.045$) (Fig. 2*D* and *E*), suggesting increased angiogenesis and wound bed vascularity. Interestingly, there was a twofold increase in $CD11b^+$ immune cells within the wound bed at day 10 in control wounds compared with those treated with 0.2% FLX, indicating that inflammation persists in the absence of FLX treatment ($P = 0.006$) (Fig. 2*F* and *G*).

Since proreparative macrophages are known to mediate both wound healing and angiogenesis by acting as cellular chaperones, we hypothesized that the topically applied FLX could be promoting the generation of proreparative macrophages at the local wound environment. This seemed likely since 5-HT has previously been shown to modulate the polarization of macrophages toward an anti-inflammatory phenotype (14). Therefore, we immune phenotyped the FLX-treated wounds using flow cytometry.

Wounds were predominantly infiltrated by cells of myeloid lineage ($CD11^+CD45^+$). $CD11b^+CD45^+$ cells were fewer in FLX-treated compared with vehicle-treated wounds at day 10, from 5.25×10^6 to 1.53×10^6 ($P = 0.016$) (Fig. 3*A* and *B*), confirming the immunohistochemistry observations. Importantly, FLX treatment decreased $Ly6C^+Ly6G^-$ inflammatory monocyte/macrophage (mo/ma) numbers overall in the wound at day 10 from 3.2 to 0.5×10^6 cells ($P = 0.032$) (Fig. 3*C* and *D*). Although there was an increase in the percentage of neutrophils (due to a decrease in absolute numbers of mo/ma at the wounds), there was no difference in the absolute counts between the two groups (Fig. 3*E*), suggesting that wound neutrophils are not affected by FLX. Moreover, within the $Ly6C^+Ly6G^-$ inflammatory mo/ma, there was a notable decrease in proinflammatory $CD206^-MHCII^+$ macrophages (from 77.9 to 26.2%, $P = 0.008$), indicating a shift away from the proinflammatory phenotype (Fig. 3*F* and *G*).

Analysis of wound beds by quantitative RT-PCR (qRT-PCR; primer sequences listed in Table 1) on day 10 showed a 1.65-fold increase in arginase 1 (Arg-1), a marker for alternatively activated, prohealing macrophages ($P = 0.04$), and 5.6-fold decrease in inducible nitric oxide synthase (Nos2), a marker for classically activated, proinflammatory macrophages ($P = 0.007$), compared with the control group, supporting the notion of a more proreparative wound environment (Fig. 4*A* and *B*). FLX-treated wounds had a twofold, 2.4-fold, and 2.2-fold decrease in *Tnf, Ifng*, and *Il6* transcript, respectively, compared with the control group, indicating that FLX decreased inflammation in the wound bed (Fig. 4*C–E*). Topical application of FLX induced the expression of *Pdgfb* (1.8-fold, $P = 0.008$) and *Col3a1* (2.3-fold, $P = 0.004$), which are essential for granulation tissue formation and wound resolution (Fig. 4*F* and *G*). Interestingly, we found a significant increase (2.2-fold, $P = 0.009$) in the *Hspa1a* transcript (Fig. 4*H*), which encodes for the HSP70, an anti-inflammatory mediator, that is downregulated in diabetic mice postwounding (15). Serotonin has been shown to induce HSP expression in the absence of heat stress (16,17). Multiple studies have demonstrated that HSP70 induces Arg-1 (18) and downregulates tumor necrosis factor (Tnf) (19), interleukin 6 (Il6), nitric oxide (20), and interferon-γ (Ifng) (16) (Fig. 4*I*), consonant with the findings in the current study.

For clinical translation of a topically administered drug, ideally, systemic absorption should be minimized to limit the side effect profile. After 10 days of daily dosing with topically applied 0.2% FLX, the levels of FLX in mouse plasma ranged from 23 to 64 ng/mL (Supplementary Fig. 3*A* and *B*), with no change in plasma

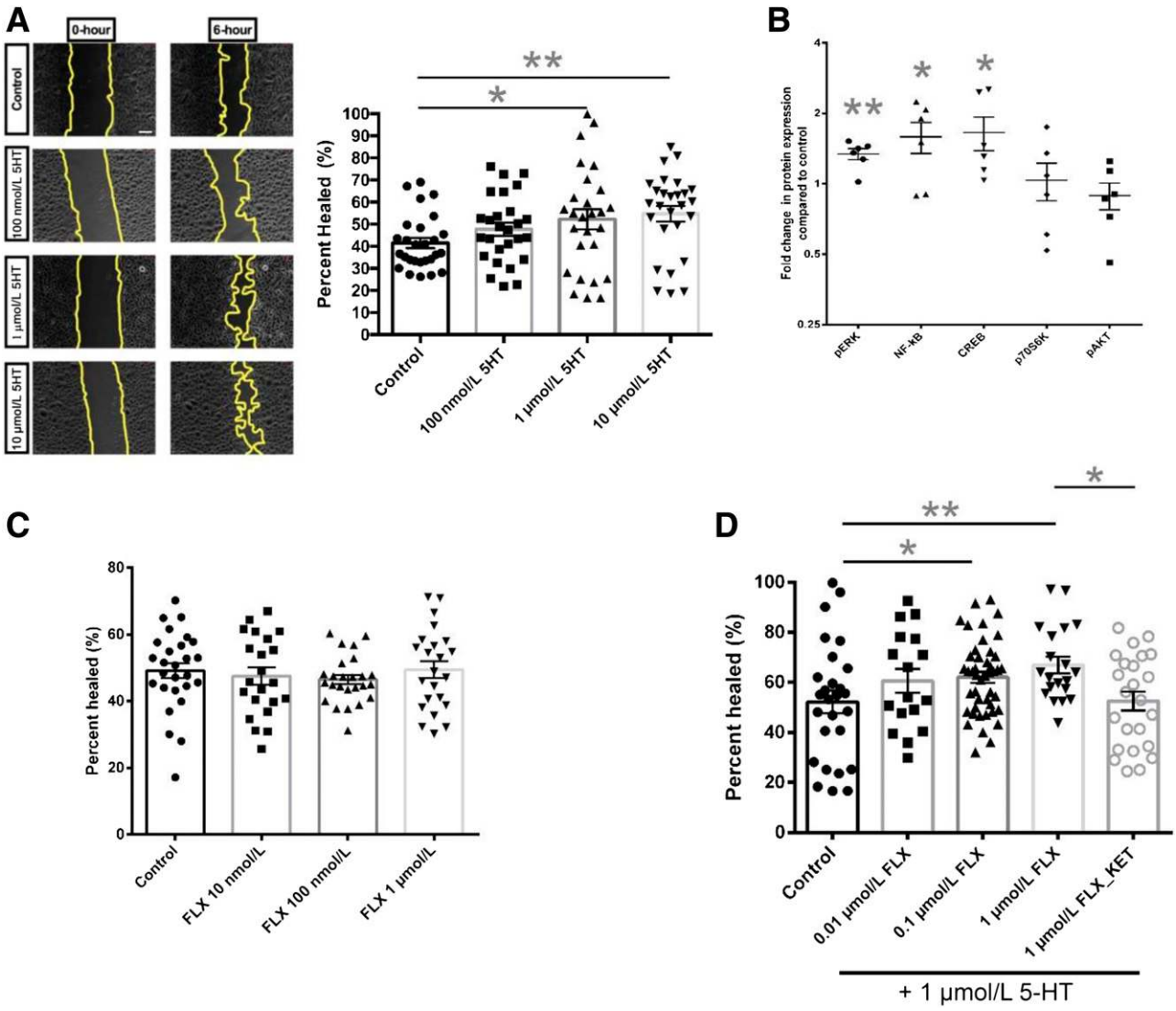

Figure 1—Serotonin and FLX increase re-epithelialization. Scratch wound assays were performed on confluent cultures of NHKs from three donors (n = 27/group) in the presence of varying doses of serotonin (5-HT). Scale bar = 100 μm (*A*). Intracellular signaling nine-plex assays; NHKs from three different donors were cultured in 100-mm dishes until 80% confluent and treated with 10 μM 5-HT for 6 h. Protein lysates (n = 6/group) were collected and quantified with Bradford assay before proceeding with Multiplex protocol as outlined in the RESEARCH DESIGN AND METHODS section. Results were normalized to total GAPDH protein expression. Fold change compared with nontreated control group were presented (*B*). Significant upregulation of phosphorylation of ERK, NF-κB, and CREB was identified. Scratch wound assays were repeated with different doses of FLX in the absence (*C*) and presence (*D*) of 5-HT. HTR2A blocker ketanserin (KET) was added to confirm the 5-HT–dependent mechanism. Data represented as mean ± SEM. Kolmogorov-Smirnov tests were performed to confirm normality in data distribution. Two-way ANOVAs with correction to multiple comparisons were used to assess statistical significance. *$P \leq 0.05$; **$P \leq 0.01$.

serotonin concentrations (Supplementary Fig. 4). The FLX levels measured are twofold lower than plasma levels in patients treated with oral FLX at therapeutic doses and are also significantly lower than levels in mice treated with neurologically therapeutic doses of FLX, either orally or intraperitoneally administered (21,22). To further query if our topical FLX treatment induces psychological effects, we performed behavioral experiments on wounded diabetic mice treated with topically applied FLX and found that the animals treated with FLX did not exhibit significant changes in their behavior in the light/dark chamber box test, a measure of anxiety (23) (Supplementary Fig. 3*C*), or in the novel object recognition test, a measure of cognition (24) (Supplementary Fig. 3*D*). These findings indicate the potential of topically delivered FLX, contrasted to other delivery methods for improving healing with minimal systemic effects.

DISCUSSION

Chronic nonhealing ulcers, commonly found in patients with diabetes, represent a significant source of morbidity and expense to both patients and the health care system in

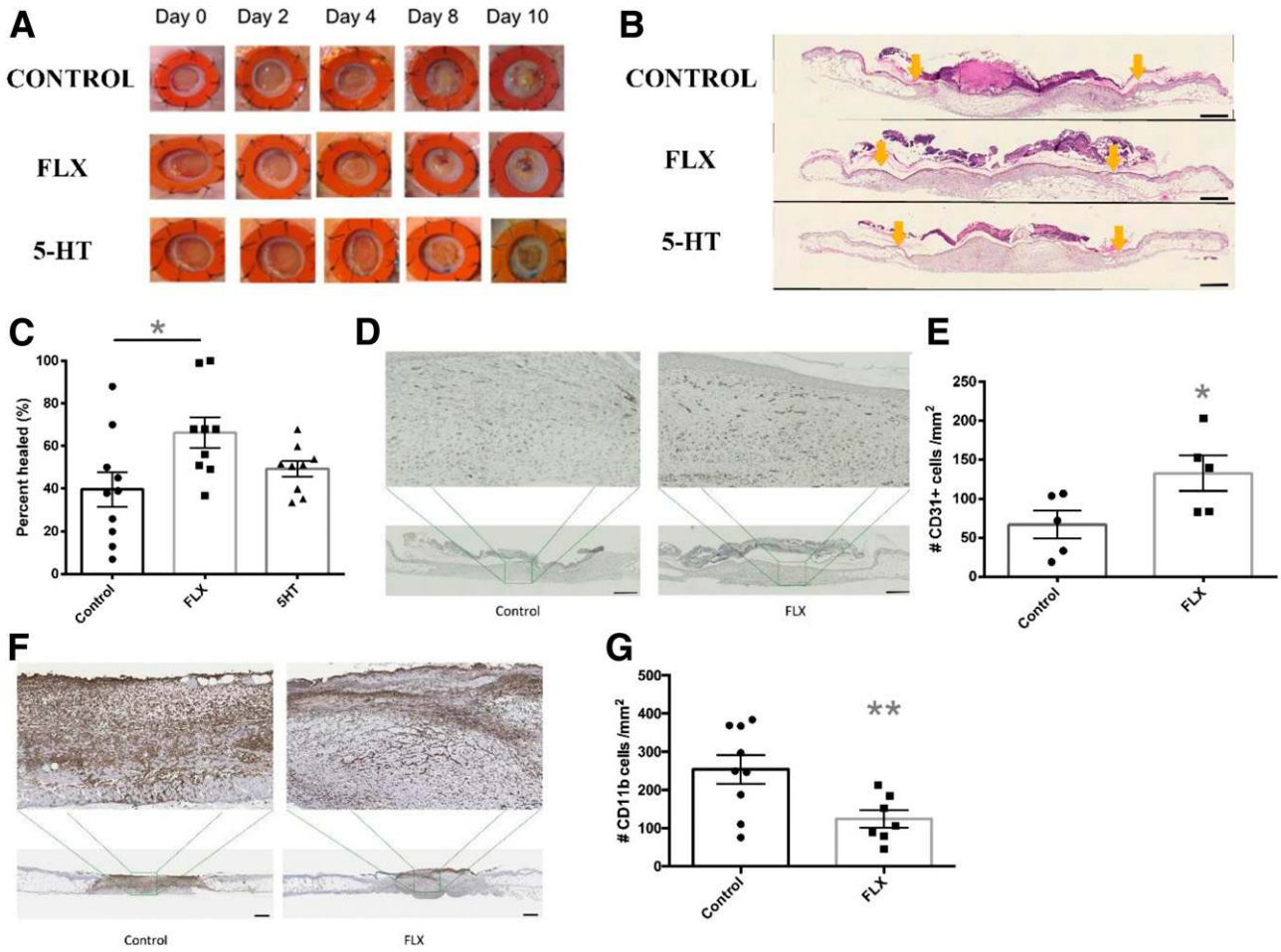

Figure 2—Representative wound images from day 0 to day 10 after excisional biopsy in *db/db* mice treated with topical PEG vehicle (control), topical 0.2% FLX, or topical serotonin (5-HT). Scale bar = 1 mm. *A*: Representative hematoxylin-eosin stains from each group for re-epithelialization analysis at day 10. Wound beds are demarcated by orange arrows (*B*). Average percent re-epithelialization per treatment group was quantified at day 10 (*C*). Immunohistochemical stainings of CD31 (*D*) and CD11b (*F*) on wound bed sections at day 10 postwounding. Average number of positive cells per treatment group was quantified (*E* and *G*). Data represented as mean ± SEM. Kolmogorov-Smirnov tests were performed to confirm normality in data distribution. Student *t* tests were used to assessed statistical significance $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

the U.S. Persistent inflammation and delayed epithelialization contribute to the stalled healing in these ulcers. Herein, we have illustrated the polypharmacological targeting in an impaired wound healing model, using a topical formulation of FLX that increases the availability of 5-HT.

Systemic administration of FLX has been shown to exhibit anti-inflammatory properties in microglia, splenocytes, and lymphocytes (8). However, the potential for local modulation of the inflammatory environment in a chronic wound by topical application of FLX has not yet been explored. Farahani et al. (25) showed that in psychologically stressed and nonstressed rats, systemically administered FLX improved healing of acute surgical wounds. However, such effects could have been due to anxiolytic and analgesic properties of FLX. In the diabetic mouse model of impaired wound healing, we have demonstrated that topical FLX improves wound healing through local effects on multiple cell types within the wound. The beneficial effects of FLX in our study appear to be independent of psychological changes.

There are some limitations to this study. Our in vivo studies are limited to only female diabetic mice. We have not taken into account the effects of sex hormones that may (26) or may not (27) impact the outcome of cutaneous wound healing. Intact male and female wild-type mice heal similarly, but gonadectomy revealed the inhibitory effects of androgens in males and the enhancing effects of estrogens (28). In humans, lower total testosterone levels commonly occur with type 2 diabetes (29), which may influence wound healing outcomes. Therefore, we chose to conduct our study in female mice only to limit potential confounding factors. Due to the limited tissue available for harvest from the wound, this study provides only gene expression data of the proposed

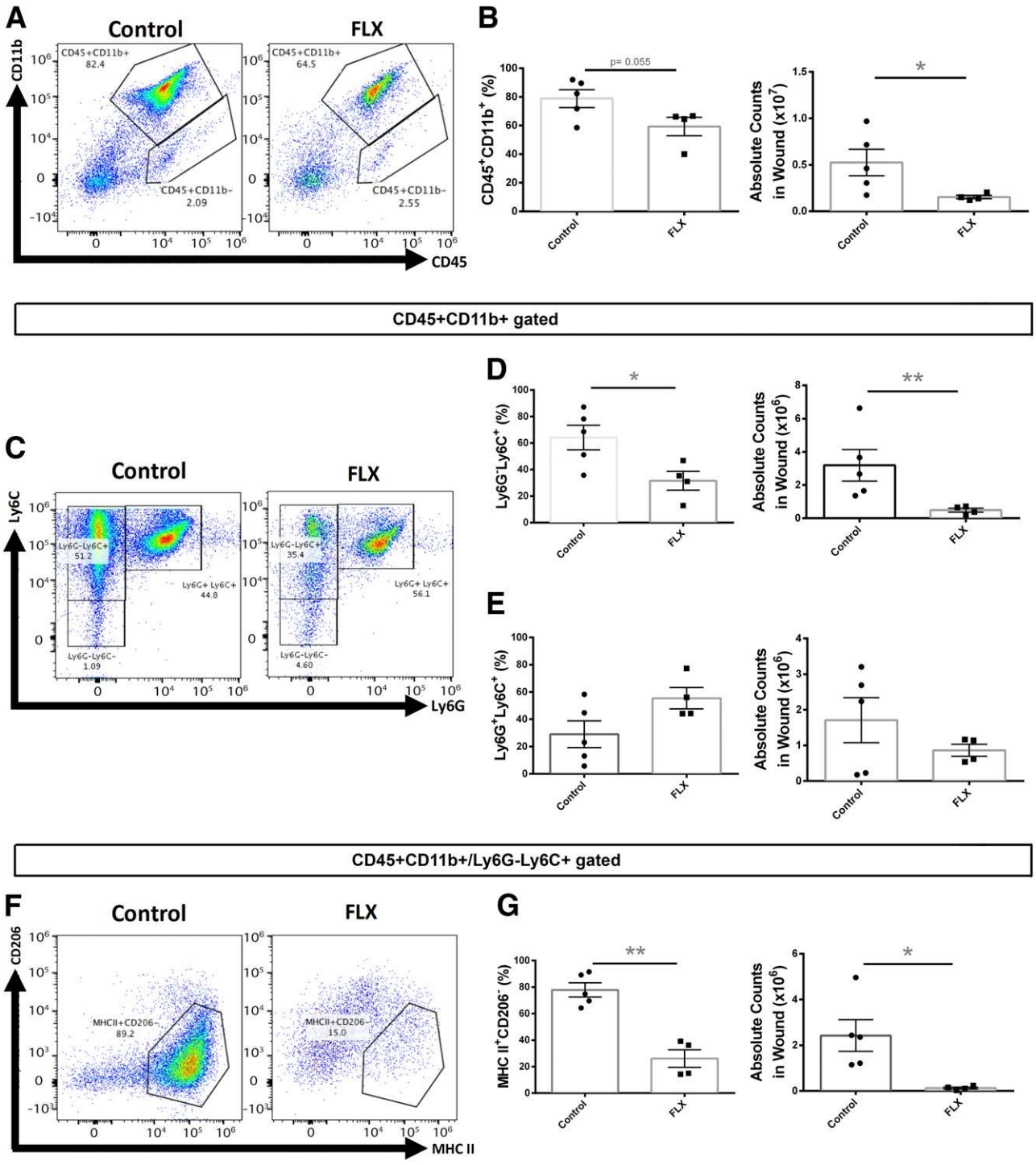

Figure 3—Topical FLX attenuates inflammation at wound site as shown by flow cytometry analysis. Wound cells were quantified based on percent, and cellularity $CD11b^+CD45^+$ myeloid cells are noted to be fewer (% and absolute number) (*A* and *B*). Within the $CD11b^+CD45^+$ population, the $Ly6C^+Ly6G^-$ inflammatory macrophages are decreased (*C* and *D*). No significant change in $Ly6C^+Ly6G^+$ neutrophil counts at wound site (*E*). Decreased numbers and percent of MHCII and $CD206^-$ cells within $Ly6C^+Ly6G$ inflammatory macrophage population (*F* and *G*). Data represented as mean ± SEM. Nonparametric Mann-Whitney *U* tests were performed to assess statistical significance. $^*P \leq 0.05$; $^{**}P \leq 0.01$.

signaling pathways at the wound site. Although gene expression is not always predictive of the protein level, it can be useful in the interpretation of the cellular and molecular events in the wound microenvironment (30,31).

Since FLX has undergone extensive toxicology profiling and safety evaluation, and postmarketing adverse event reporting, the path to translation to clinic for this novel indication could be shortened, and a therapeutic need filled rapidly. We believe that this topical therapeutic represents

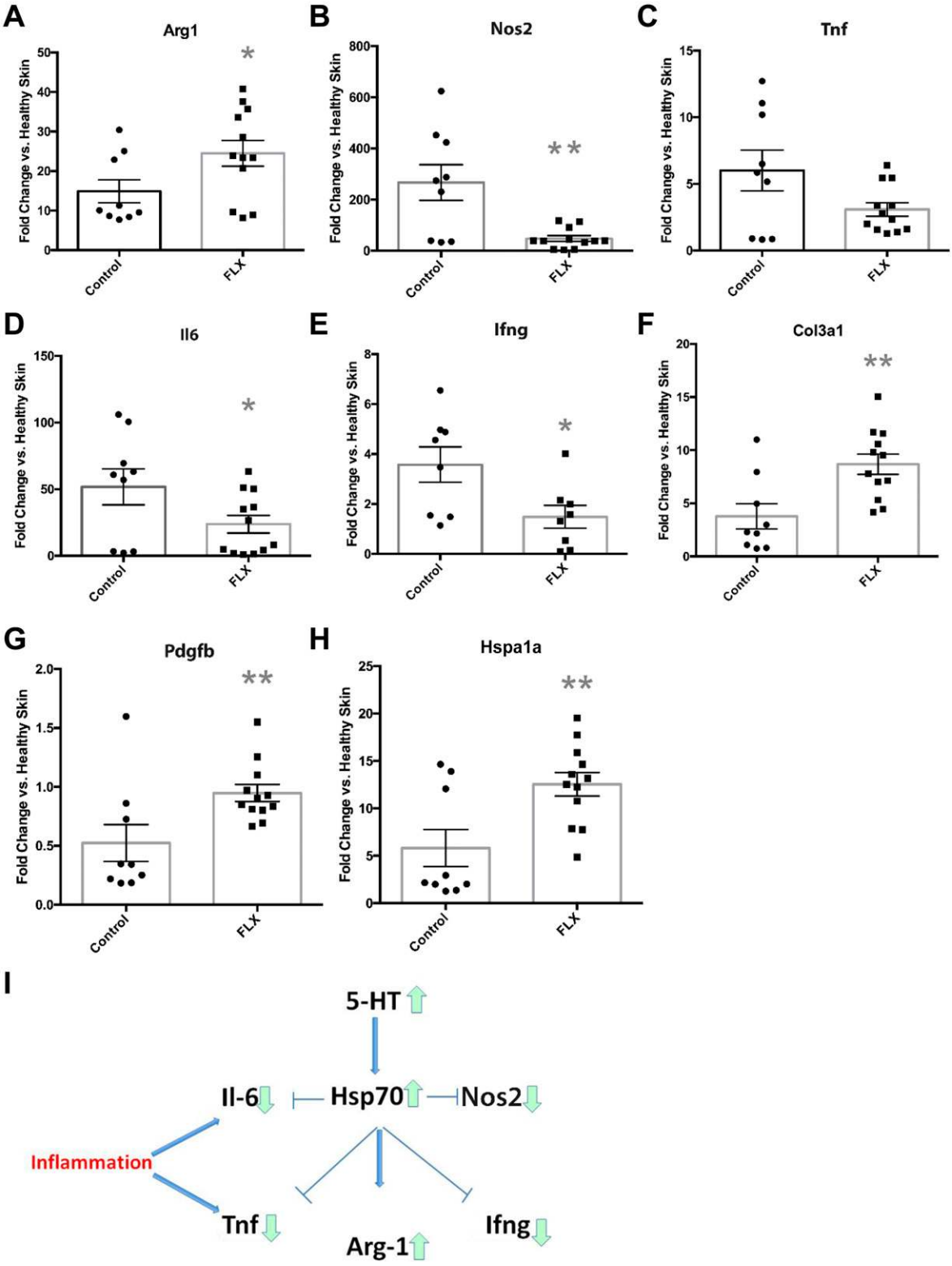

Figure 4— Topical FLX induces gene expression in Hsp70 signaling pathways. qRT-PCR was performed on RNA extracted from flash frozen skin tissues. Fold change in gene expression compared with healthy nonwounded skin was shown for Arg-1 (*A*), inducible NOS (iNOS; Nos2) (*B*), Tnf (*C*), Il6 (*D*), Ifng (*E*), collagen type III α (Col3a1) (*F*), platelet-derived growth factor β (Pdgfb) (*G*), and heat shock 70 kDa protein 1A (Hspa1a) (*H*). Taken together, a signaling pathway for serotonin in cutaneous inflammation was proposed (*I*). Data represented as mean ± SEM. Nonparametric Mann-Whitney *U* tests were performed to assess statistical significance. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

Table 1—Primer sequences

Gene target	Forward	Size (bases)	Reverse	Size (bases)
Gapdh	AGGTCGGTGTGAACGGATTTG	21	TGTAGACCATGTAGTTGAGGTCA	23
18s	CCCAACTTCTTAGAGGGACAAG	22	GCTTATGACCCGCACTTACT	20
Tbp	GTTTCTGCGGTCGCGTCATTT	21	TGGGTTATCTTCACACACCATGAA	24
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	22	AGGAGCTGTCATTAGGGACA	20
iNos	GTTCTCAGCCCAACAATACAAGA	23	GTGGACGGGTCGATGTCAC	19
Tnf	CCAGACCCTCACACTCAGATC	21	CACTTGGTGGTTTGCTACGAC	21
Il6	TAGTCCTTCCTACCCCAATTTCC	23	TTGGTCCTTAGCCACTCCTTC	21
Ifng	ATGAACGCTACACACTGCATC	21	CCATCCTTTTGCCAGTTCCTC	21
Col3a1	ACGTAGATGAATTGGGATGCAG	22	GGGTTGGGGCAGTCTAGTG	19
Pdgf-bb	AAGTGTGAGACAATAGTGACCCC	23	CATGGGTGTGCTTAAACTTTCG	22
Hsp70	TGGAGATCATCGCCAACGACC	21	TCCTCCACGAAGTGGCTCACC	21

a safe alternative for the challenging clinical problem of chronic, nonhealing wounds in patients with diabetes.

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Duality of Interest. C.M.N., D.M.T., and R.R.I. have a pending patent on the use of FLX in wound healing. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. C.M.N. and D.M.T. proposed experiments, coordinated the work, and prepared the manuscript. C.M.N., D.M.T., M.D.B., M.S., D.F., J.S., and A.A. performed in vivo experiments. C.M.N., M.D.B., A.V.N., and A.M.S. performed flow cytometry experiments. C.M.N. and D.F. performed qRT-PCR experiments. C.M.N., D.N., and B.H. performed scratch wound assays. A.G. performed high-performance liquid chromatography and analyses. D.D. and M.G.G. performed behavioral experiments. J.J.F. and R.W.C. gave early critical input. R.R.I. conceived and oversaw the study and critically edited the manuscript. R.R.I. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. This study was presented at the 29th Annual Meeting of the Wound Healing Society, San Diego, CA, 5–9 April 2017, and the 76th Annual Meeting of the Society of Investigative Dermatology, Portland, OR, 26–29 April 2017.

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