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STEM CELLS TRANSLATIONAL MEDICINE

ID1/ID3 mediate the contribution of skin fibroblasts to local nerve regeneration through Itga6 in wound repair

Zelin Chen | Gufang Shen | Xu Tan | Langfan Qu | Can Zhang | Le Ma | Peng Luo | Xiaohui Cao | Fan Yang | Yunsheng Liu | Yu Wang | Chunmeng Shi

Institute of Rocket Force Medicine, State Key Laboratory of Trauma, Burns and Combined Injury, Army Medical University, Chongqing, People's Republic of China

Correspondence
Chunmeng Shi, PhD and Yu Wang, PhD, Institute of Rocket Force Medicine, State Key Laboratory of Trauma, Burns and Combined Injury, Army Medical University, Chongqing 400038, People's Republic of China.
Email: shicm@sina.com (C. S.) and
Email: wangyusmmu@163.com (Y. W.)

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Abstract

Cutaneous wound healing requires intricate synchronization of several key processes. Among them, local nerve regeneration is known to be vitally important for proper repair. However, the underlying mechanisms of local nerve regeneration are still unclear. Fibroblasts are one of the key cell types within the skin whose role in local nerve regeneration has not been extensively studied. In our study, we found skin fibroblasts were in tight contact with regenerated nerves during wound healing, while rare interactions were shown under normal circumstances. Moreover, skin fibroblasts surrounding the nerves were shown to be activated and reprogrammed to exhibit neural cell-like properties by upregulated expressing inhibitor of DNA binding 1 (ID1) and ID3. Furthermore, we identified the regulation of integrin α6 (Itga6) by ID1/ID3 in fibroblasts as the mechanism for axon guidance. Accordingly, transplantation of the ID1/ID3-overexpressing fibroblasts or topical injection of ID1/ID3 lentivirus significantly promoted local nerve regeneration and wound healing following skin excision or sciatic nerve injury. Therefore, we demonstrated a new role for skin fibroblasts in nerve regeneration following local injury by directly contacting and guiding axon regrowth, which might hold therapeutic potential in peripheral nerve disorders and peripheral neuropathies in relatively chronic refractory wounds.

KEYWORDS
fibroblasts, nerve, regeneration, skin

Significance statement

The present study showed that fibroblasts are activated and reprogrammed to a neural cell-like state following wounding and further have direct contact with local nerves to promote axon regrowth through the ID1/ID3-Itga6 pathway. Transplantation of ID1/ID3 fibroblasts significantly improves local nerve regeneration following wounding. The results of the present study increase the current understanding of the pathophysiology of local nerve injury and may aid the

Zelin Chen and Gufang Shen contributed equally to this work.

clinical challenge of treating chronic refractory wounds resulting from traumatic and pathologic impairment of peripheral innervation and peripheral nerve regeneration.

1 | INTRODUCTION

Skin, the largest organ of the body, easily suffers from various injuries throughout life. Tissue repair mechanisms of skin have been studied for decades and have highlighted that several key processes are required for rapid wound closure.[1,2] Among them, neural responses following wounding have been shown to greatly contribute to wound healing.[3] Both pathologic and traumatic impairment of peripheral innervation result in chronic tissue repair and even failure to heal.[4,5] Subsequent studies indicated that the contribution of skin innervation to wound healing occurred mainly by axonal sprouting of neurons and their relative growth factors being released into the wound tissues.[6-8] Moreover, restoration of pain, temperature, and touch perceptions is necessary for high-quality wound healing. However, the underlying mechanisms of local nerve regeneration following wounding are still unclear.

Previous studies have shown that nerve-surrounding cells, including Schwann cells and macrophages, are rapidly activated to repair nerves.[9] Schwann cells have been broadly studied and have been shown to be involved in several main processes of nerve repair, for instance, neurotrophic factor production, nerve remyelination, and recruitment of macrophages to clean up debris.[6,10,11] However, fibroblasts, one of the key cell types in the skin, have not been extensively studied in nerve injury and regeneration. According to previous studies, fibroblasts quickly respond to damage and participate in initial clearing of nerve tissue debris. They also interact with Schwann cells to help form bands of Büngner to initiate the nerve regenerative response and produce extracellular matrix (ECM) molecules to support neurite extension.[12] In Simona Parrinello's study,[13] following sciatic nerve injury, fibroblasts were shown to mediate Schwann cell sorting through ephrin-B/EphB2 interactions and further induce directional migration to guide axon sprouting. According to a recent study,[14] the regenerative axons were not myelinated, which suggested other cells besides Schwann cells might directly contact the axons for regrowth. Whether the fibroblasts directly guide axon regrowth following wounding is still largely unknown. Interestingly, in this study, skin fibroblasts were found to be in tight contact with local injured nerves during wound healing, while rare interactions were observed under normal circumstances. Further, skin fibroblasts near the injured nerves were shown to upregulate the expression of inhibitor of DNA binding 1 (ID1)/ID3, which induced the skin fibroblasts to exhibit neural cell-like properties. More importantly, ID1/ID3 regulated the expression of integrin α6 (Itga6), which was shown to mediate interactions between axons and skin fibroblasts to promote axon extension. We administrated ID1/ID3-overexpressing fibroblasts or ID1/ID3 lentiviruses topically to skin wound tissues, and both cases showed increased local nerve regeneration and accelerated wound healing. More importantly, transplantation of ID1/ID3-overexpressing fibroblasts significantly improved sciatic nerve regeneration following transection. In summary, our results demonstrated that skin fibroblasts were activated to directly contact injured nerves and contribute to axon sprouting regulation through the ID1/ID3-Itga6 pathway, which might shed new light on treatments for peripheral nerve injury and peripheral neuropathies of relatively chronic refractory wounds.

2 | MATERIALS AND METHODS

2.1 | Animals and wound models

Male and female Sprague Dawley (SD) rats (6-10 weeks old) were used as sciatic nerve injury models. C57/BL mice (6-10 weeks old) were used as radiation combined with cutaneous wound models. Pdgfr-α^{Cre}:Ai9 mice derived from crossing Pdgfr-α^{Cre} mice with Ai9 mice (both were purchased from Jackson Laboratory) and C57/BL mice (6-10 weeks old) were used for ear punch injury models. Newborn C57/BL mice were used for skin fibroblast isolation. In vivo experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Army Medical University (AMU), and all procedures were approved by the Animal Care and Use Committee of the AMU. Dorsal skin wound models and radiation combined skin wound models were used previously.[15] Ear punch injury models were described previously.[14] Briefly, mice were punched in the center of the ear pinnae using a sterile stainless steel 2-mm hole punch. Wound healing images of each mouse skin wound and ear punch wound and wound tissues were captured at indicated time points following wounding. Sciatic nerve injury models were used previously.[16] In short, SD rats were anesthetized with 1% pentobarbital (30 mg/kg). A 5 mm gap was created in the sciatic nerve, and the gap was bridged using a silicon conduit. The injured sciatic nerves were harvested at 6 weeks following wounding.

2.2 | Cell isolation

The isolation of neonatal mouse fibroblasts was described previously.[17] Previous isolated primary human skin fibroblasts were used, and the protocol was approved by the ethics committee of the AMU. Cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Beyotime). Dorsal root ganglion (DRG) neuron isolation was done as follows.[18] Dorsal root ganglia at lumbar levels 4 to 5 were dissected from adult rats and cut into small pieces. They were then digested with 0.25% collagenase I (Worthington) and 0.01% trypsin at 37°C for 40 minutes with shaking. Cells were then passed through a 75-μm cell strainer and resuspended in Neurobasal Medium (12348017, Gibco) with 2% B27 (12587010, Gibco) and L-glutamine (2 mM, Gibco).

2.3 | ID1/ID3 overexpression in fibroblasts

After human and mouse fibroblasts were grown to 30% to 50% confluence, cells were transfected in Opti-MEM Reduced Serum Media (Invitrogen) with lentiviruses (Gene-Pharma) encoding for mCherry, mCherry + ID1, or mCherry + ID3, according to the manufacturer's instructions. Six hours later, the medium was replaced with fresh culture medium. Following 7 days of culturing, successfully transfected mCherry positive cells were sorted with a Beckman Moflo XDP flow cytometer with 561 nm excitation and 650 nm emission. Sorted cells were cultured for further experiments.

2.4 | Cell transplantation

2.4.1 | ID1/ID3-overexpressing mouse fibroblasts transplanted to the mouse skin wound model

After radiation combined with dorsal skin wounding, 60 mice were randomly divided into four groups, namely, the phosphate buffer saline (PBS) group, control fibroblasts group, ID1 fibroblasts group, and ID3 fibroblasts group. A total of 0.2 mL PBS alone, PBS with 2×10^6 control fibroblasts, or ID1 or ID3 overexpression fibroblasts were topically injected into the subcutaneous tissues around the wound edges. Images of the wound areas were captured at indicated time points. Wound tissues from three mice in each group at each time point were harvested at 7, 19, and 28 days following wounding. The granulation tissues growth, wound scar area, scar depth, the ECM deposition, and the expression of α-smooth muscle actin (α-SMA) and tuj1 were analyzed.

2.4.2 | ID1/ID3-overexpressing mouse fibroblasts transplanted to the rat sciatic nerve injury model

After sciatic nerve injury, 24 rats were randomly divided into four groups, namely, the PBS group, control fibroblasts group, ID1 fibroblasts group, and ID3 fibroblasts group. A total of 0.1 mL PBS alone, PBS with 2×10^6 control fibroblasts, or ID1 or ID3 overexpression fibroblasts were injected into the silicon conduit. Six weeks later, whole nerve tissues were harvested from the proximal to distal segments to evaluate nerve regeneration using histological and immunofluorescent staining. The gastrocnemius muscles of both legs were also harvested, and the relative gastrocnemius muscle weight (RGMW) was measured. The muscle tissues were fixed with 4% polyoxymethylene for further histological staining.

2.4.3 | ID1/ID3-overexpressing lentivirus transfection in ear punch models

After ear punch injury, 63 mice were randomly divided into three groups, namely, the control lentivirus group, ID1 overexpression lentivirus group, and ID3 overexpression lentivirus group. A total of 1×10^7 U lentivirus in 50 μL culture medium was subcutaneously injected around the punch injury. The images of the hole area were captured at indicated time points. Wound ears from three mice in each group per time point were harvested at 7, 14, 21, 35, and 43 days following wounding and fixed by 4% polyoxymethylene for further histological staining.

2.5 | Scratch migration assay

Control or ID1/ID3-overexpressing mouse fibroblasts were seeded into six-well plates and grown to 100% confluence for another day. A p200 pipette tip was used to make a straight line through the cell sheet in each well. The wells were then replaced with low serum (2%) culture medium following clearing away of cell debris. The images of the scratch wound at the indicated position were taken at 0, 6, 12, and 24 hours following scratching. The images were processed with ImageJ software. Three independent experiments were performed for each group.

2.6 | Scratch wound model

Control or ID1/ID3-overexpressing human fibroblasts were seeded into 24-well plates with cell climbing slices and grown to 100% confluence for another day. A p200 pipette tip was used to make a straight line through the cell sheet in each well. After clearing away the cell debris, the wells were replaced with fresh culture medium. The cells were fixed with 4% paraformaldehyde at indicated time points for further experiments.

2.7 | Statistical analysis

SPSS 13.0 software was used for statistical analysis. Data were expressed as the mean ± SD. Comparisons of multiple groups were performed using a one-way analysis of variance. $P < .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | Skin fibroblasts associate with peripheral nerves during wound healing

In normal skin, peripheral nerves are distributed in a dense reticular manner (Figure S1A). Following dorsal full-thickness injury, the regeneration of the skin peripheral nerves began at about 7 days (Figure 1A), which is when the skin fibroblasts were also activated.[19] To examine whether the skin fibroblasts contribute to the regeneration of the injured local nerves, we crossed the platelet-derived growth factor receptor-α (Pdgfr-α, the pan-fibroblast marker[20])^{Cre} transgenic mice with the Ai9 reporter mice to obtain Pdgfr-α^{Cre}:Ai9

mice to label the skin fibroblasts. In the Pdgfr-α^{Cre}:Ai9 mice ear punch model, lesioned axons (staining the pan-neuronal marker, class III beta-tubulin [Tuj1])[21] began to sprout to form a tight, interlinked nervous network around the wound margin at 7 days after wounding (Figure 1B). This axon network was densely populated by tdTomato positive skin fibroblasts (Figure 1B). High optical magnification revealed intimate contact between tdTomato positive skin fibroblasts and nerves (Figure 1C). In the dorsal skin wound model, results of costaining Pdgfr-α with Tuj1 showed both larger nerve bundles (Figure 1D) and single nerve fibers (Figure 1E) that were tightly surrounded by skin fibroblasts in the wound tissues. High optical magnification and three-dimensional reconstruction also showed tight contact between Pdgfr-α positive skin fibroblasts and nerves (Figure 1F,G). Rare associations between fibroblasts and nerves were observed in normal skin tissues (Figure S1B). These data indicated that skin fibroblasts associated with peripheral nerves during wound healing and they might play important roles in the regeneration of peripheral nerves.

3.2 | ID1/ID3 are upregulated in skin fibroblasts near nerves following wounding

To explore the underlying mechanism of the above phenomenon, we first reanalyzed our previous whole gene expression profile of wound activated skin fibroblasts[15] and identified the genes of ID proteins, especially ID1 and ID3, as the most abundant significantly upregulated genes (Table S1). As in previous reports, ID1 and ID3 showed overlapping expression patterns and shared similar biochemical behavior in several biological processes including neurogenesis.[22,23] Thus, we further assessed whether the ID1/ID3 in skin fibroblasts near the nerves contributed to nerve regeneration following wounding. Data of the dorsal skin wound model showed that expression of ID1/ID3 was increased at 3 days following wounding and was sustained to the remodeling phase (>15 days; Figure 2A,B). Results of costaining ID1/ID3 with Pdgfr-α showed that most ID1/ID3 positive cells were dermal fibroblasts (Figure 2E,F). The expression of ID1/ID3 in fibroblasts was also upregulated in the mechanical scratch wound model in vitro (Figure 2C, D,G,H). More importantly, we costained tuj1 with ID1 or ID3 in skin wound tissues and found that skin fibroblasts surrounding both larger nerve bundles and single nerve fibers were ID1/ID3 positive (Figures 2I, J and S1C,D). The above data indicated that ID1/ID3 might mediate the promotion effects of skin fibroblasts to nerve regeneration.

3.3 | ID1/ID3 regulate the neural cell-like properties of skin fibroblasts

Next, we assessed the neural relative properties of skin fibroblasts using ID1/ID3 transduction experiments. Skin fibroblasts with ID1/ID3 overexpression (Figure S2A,B) exhibited increased colony-forming ability (Figures 3A and S2C) and migration ability (Figure 3B). Moreover, we tested the expression of neural stem cell markers Sox2 and Nestin, the Schwann cell marker S100β, the astrocyte marker Gfap, and mesoderm markers α-SMA and collagen 1A1 in ID1/ID3-overexpressing skin fibroblasts. Western blot and immunofluorescence results showed upregulated expression of Sox2, Nestin, S100β, and Gfap (Figure 3C,D); however, there was decreased expression of α-SMA and collagen 1A1 (Figure S2D,E), which indicated that ID1/ID3 induced skin fibroblasts to exhibit neural cell-like properties and lose mesoderm properties.

3.4 | ID1/ID3 regulate Itga6 expression in fibroblasts to promote axonal regeneration

To demonstrate the underlying mechanism of ID1/ID3 in nerve regeneration promoting plasticity of fibroblasts, we performed RNA sequencing of the ID1/ID3-overexpressing human skin fibroblasts. About 197 genes in ID1-overexpressing fibroblasts and 161 genes in ID3-overexpressing fibroblasts showed significant differential expression (Figures 4A and S3A). Among them, 131 genes were overlapped between ID1- and ID3-overexpressing fibroblasts (Figure 4B), which indicated similar regulation effects of ID1 and ID3 in skin fibroblasts. Gene ontology enrichment analysis identified terms associated with cell adhesion and biologic adhesion, as well as axon guidance and neuron projection in both ID1- and ID3-overexpressing fibroblasts (Figures 4C,D and S3B,C). Among the upregulated genes, Itga6 was the most abundantly expressed gene (Figure 4E). Both real-time polymerase chain reaction (PCR) and Western blot data further confirmed the upregulation of Itga6 in ID1/ID3-overexpressing skin fibroblasts (Figure 4F,G). Knockdown of ID1/ID3 (Figure S3D-F) significantly reduced Itga6 expression (Figure 4H), as well as S100β and Gfap (Figure 4I).

Itga6 was previously reported to play an important role in axon guidance.[18] Thus, we isolated the DRG neurons and cocultured them with skin fibroblasts. Images of immunofluorescence showed the Itga6 positive fibroblasts were in close contact with DRG neurons (Figure 4J). The neurites were significantly elongated when cocultured with ID1/ID3-overexpressing fibroblasts (Figure 4K), while the neurite promotion effects of the ID1/ID3-overexpressing fibroblasts were eliminated when treated with Itga6 antibodies (Figure 4M). The neurite length was also decreased when the skin fibroblasts were transfected with small interferring RNAs (siRNAs) targeting ID1/ID3 prior to coculture (Figure 4N). All the above indicated that ID1/ID3 mediated the promotion of axon guidance in skin fibroblasts through Itga6.

3.5 | ID1/ID3 accelerate regeneration of the local nerves following ear punch wounding

Based on the above results, we next injected the ID1/ID3 lentiviruses topically around the ear punch wound margin to investigate whether ID1/ID3 would promote local nerve regeneration. Following in vivo transduction, the expression of ID1/ID3 was significantly increased in skin tissue cells, especially skin cells at 7 days and even at 43 days (Figure S4A,B). ID1/ID3 lentivirus administration accelerated the healing rate of the ear wound (Figure S5A). Very early and rapid re-

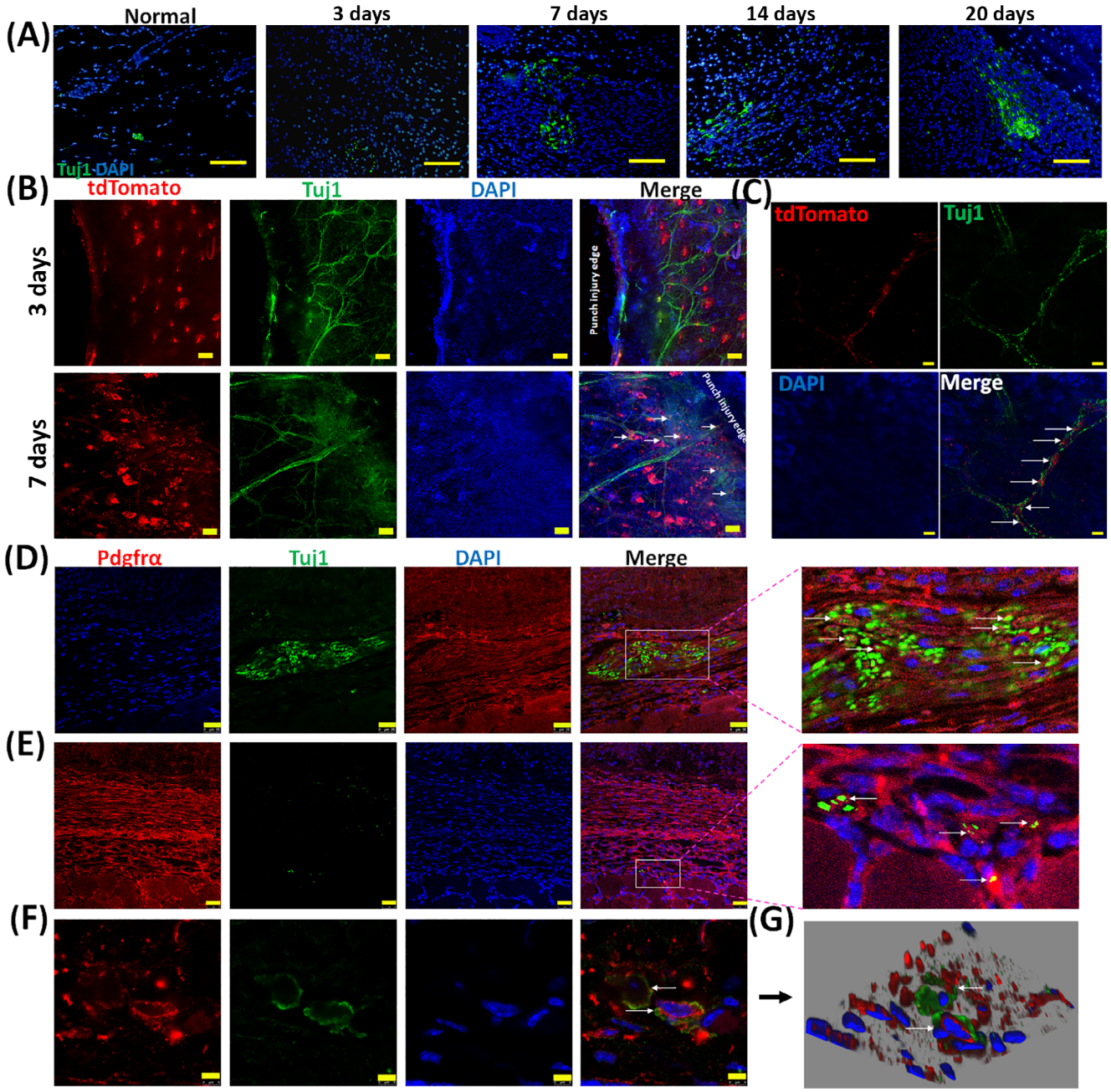

FIGURE 1 Skin fibroblasts associate with peripheral nerves during wound healing. A, The regeneration of peripheral nerves following mouse skin wound healing by staining Tuj1. B,C, Association of peripheral nerves (Tuj1 staining) and fibroblasts (tdTomato) in the wound edges following ear punch wound in Pdgfr-α^{Cre}:Ai9 mouse. Association of fibroblasts (Pdgfr-α staining) with nerve fascicles, D, or small nerve ending, E, (Tuj1 staining) in 14 days following mouse skin wound healing. F, Magnified and, G, three-dimensional images of association of the fibroblasts (Pdgfr-α staining) and novel nerve tissue (Tuj1 staining) during wound healing. Scale bar = 50 μm in (A), 100 μm in first row of (B), 150 μm in second row of (B), 10 μm in (C), 25 μm in (D, E), and 5 μm in (F)

epithelialization was observed in ID1/ID3 lentivirus administration ear wounds (Figure S5B). Moreover, growth of the blastema in the ID1/ID3 lentivirus administration ear was more robust (Figure S5B). Results of Tuj1 staining of the wound tissues at 35 days postwounding revealed significantly increased nerve fiber regeneration in ID1/ID3 lentivirus administration ear wounds (Figure S5C,D). These data indicated that ID1/ID3 could promote local nerve regeneration and wound healing in ear punch injuries.

3.6 | ID1/ID3-overexpressing fibroblasts promote the regeneration of local nerves following skin wounding

To further investigate the roles of ID1/ID3 in fibroblasts to promote nerve regeneration, we administrated ID1/ID3-overexpressing fibroblasts topically around the wound margin in a mouse model of 5 Gy total body irradiation combined with a dorsal skin wound. The wound

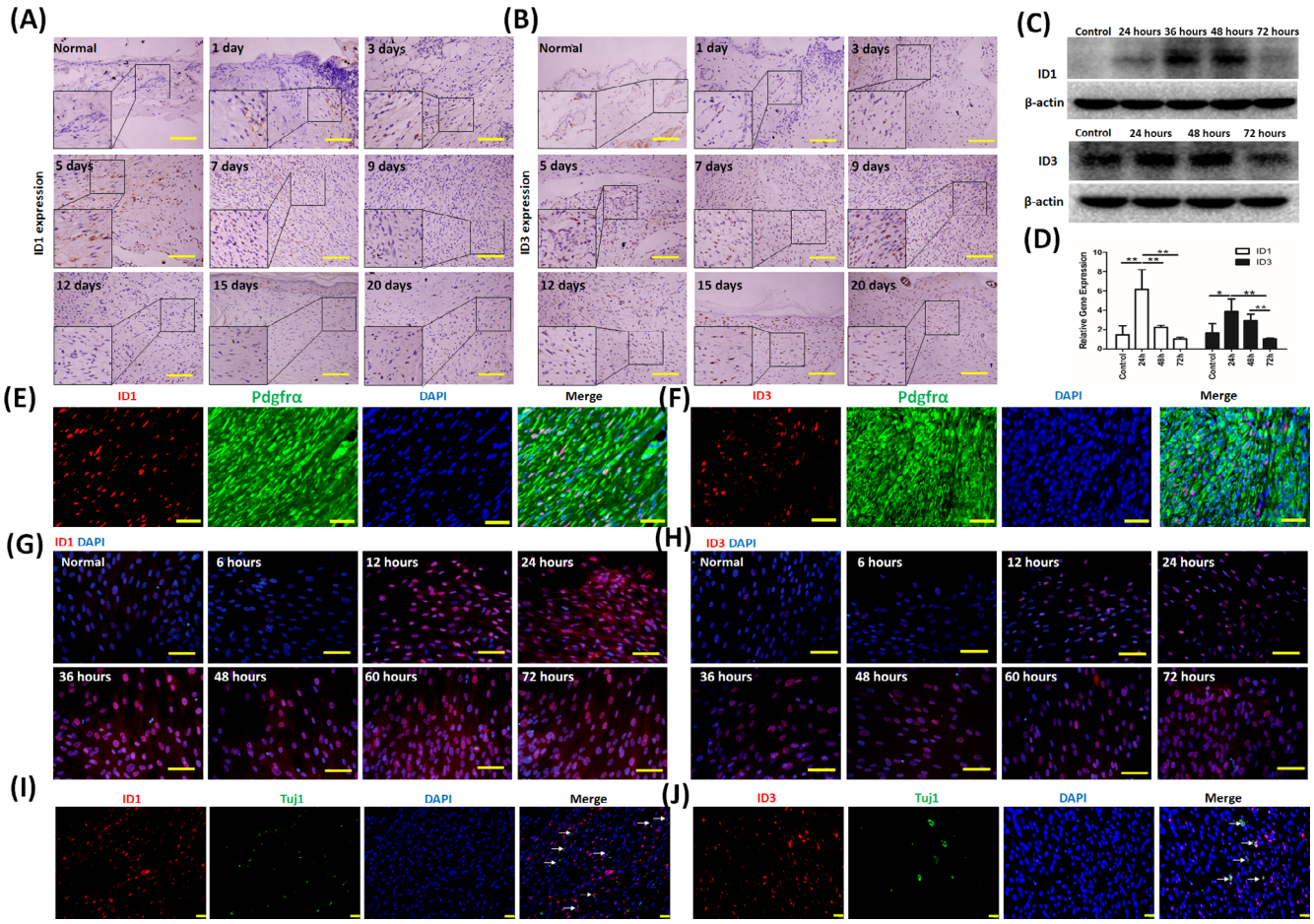

FIGURE 2 ID1 and ID3 are upregulated in skin fibroblasts following wounding. The expression of, A, ID1 and, B, ID3 at different time point following mouse skin wounding. The protein and gene expression of, C, ID1 and, D, ID3 in human skin fibroblasts following scratch wounding. Costaining of, E, ID1 or, F, ID3 with Pdgfr-α in the mouse dorsal wound tissues 7 days following wounding. Staining of, G, ID1 and, H, ID3 in human skin fibroblasts following scratch wounding. Costaining of, I, ID1 and, J, ID3 with regenerative nerve tissue (Tuj1) in 14 days following mouse skin wound tissues. Scale bar = 50 μm in (A, B, G, H), 30 μm in (E, F), and 20 μm in (I, J). Data are mean ± SD. $^{*}P < .05$; $^{**}P < .01$

healing rate was significantly accelerated in ID1/ID3-overexpressing fibroblast transplantation groups (Figure 5A), especially at the proliferation phase (7-12 days postwounding), which was the same time as nerve regeneration (Figure 1A). The proliferation of granulation tissues was also increased in ID1/ID3-overexpressing fibroblast transplantation groups at 7 days postwounding (Figure 5D). Results of wounds in remodeling phases (19 and 28 days postwounding) revealed significantly reduced scar areas, scar depths, ECM deposition, and α-SMA expression after transplantation with ID1/ID3-overexpressing fibroblasts (Figures 5B,C,E-G and S6A,B). More importantly, nerve regeneration was also increased in the transplantation groups (Figures 5H and S6C). Notably, increased nerve regrowth was found around the transplanted ID1/ID3-overexpressing cells (Figure S7A). As expected, some of the transplanted ID1/ID3-overexpressing fibroblasts expressed the Schwann cell marker S100β (Figure S7B) and astrocyte marker Gfap (Figure S7D); however, the neural stem cell marker Nestin was rare (Figure S7C). All the above data suggested that ID1/ID3 was able to increase the contribution of fibroblasts to local nerve regeneration and skin wound healing.

3.7 | ID1/ID3-overexpressing fibroblasts promote the regeneration of injured sciatic nerves

Next, we tested the therapeutic potential of cell-based therapy in peripheral nerve injury by applying ID1/ID3-overexpressing fibroblasts into the nerve conduit. Six weeks after sciatic nerve injury, we recorded the footprints to quantify functional recovery and harvested the nerve tissues for histological evaluations. The sciatic function index (SFI) values of the ID1/ID3-overexpressing fibroblast groups were significantly higher than that of the control fibroblasts and PBS groups (Figure S8A,B). Gross morphology and images of hematoxylin-eosin staining showed a larger diameter of regenerated nerves in rats receiving transplantation of ID1/ID3-overexpressing fibroblasts (Figure 6A-C). The diameter and thickness of regenerated

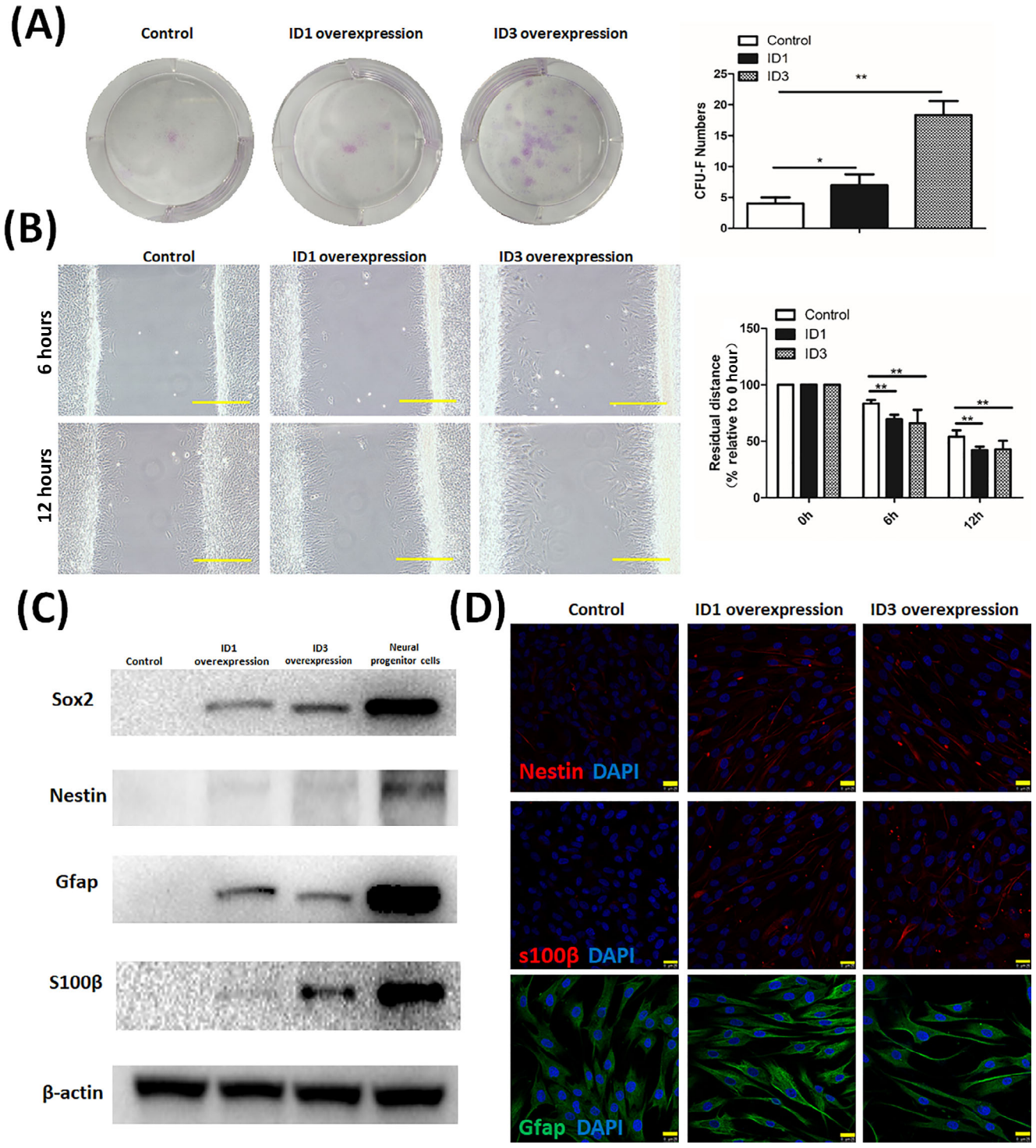

FIGURE 3 ID1/ID3 regulate the neural cell-like properties of the skin fibroblasts. A, The colony forming ability and, B, migration ability of the mouse skin fibroblasts following ID1/ID3 overexpression. C, The protein expression and, D, immunostaining of neural cell related cell markers in ID1/ID3-overexpressing human fibroblasts in vitro. Scale bar = 500 μm in (B) and 25 μm in (D). Data are mean ± SD. *$P < .05$; **$P < .01$

myelinated nerve fibers were evaluated by transmission electron microscopy. Results showed that the diameter and thickness of regenerated myelinated nerve fibers in the ID1/ID3-overexpressing fibroblast groups were greater than those in the control fibroblast group and PBS group (Figures 6D and S8C,D). In the regenerated nerve tissues, ID1/ID3-overexpressing fibroblasts survived more than control fibroblasts (Figure S9). Results of Tuj1 staining showed that ID1/ID3-overexpressing fibroblasts in the regenerated nerves

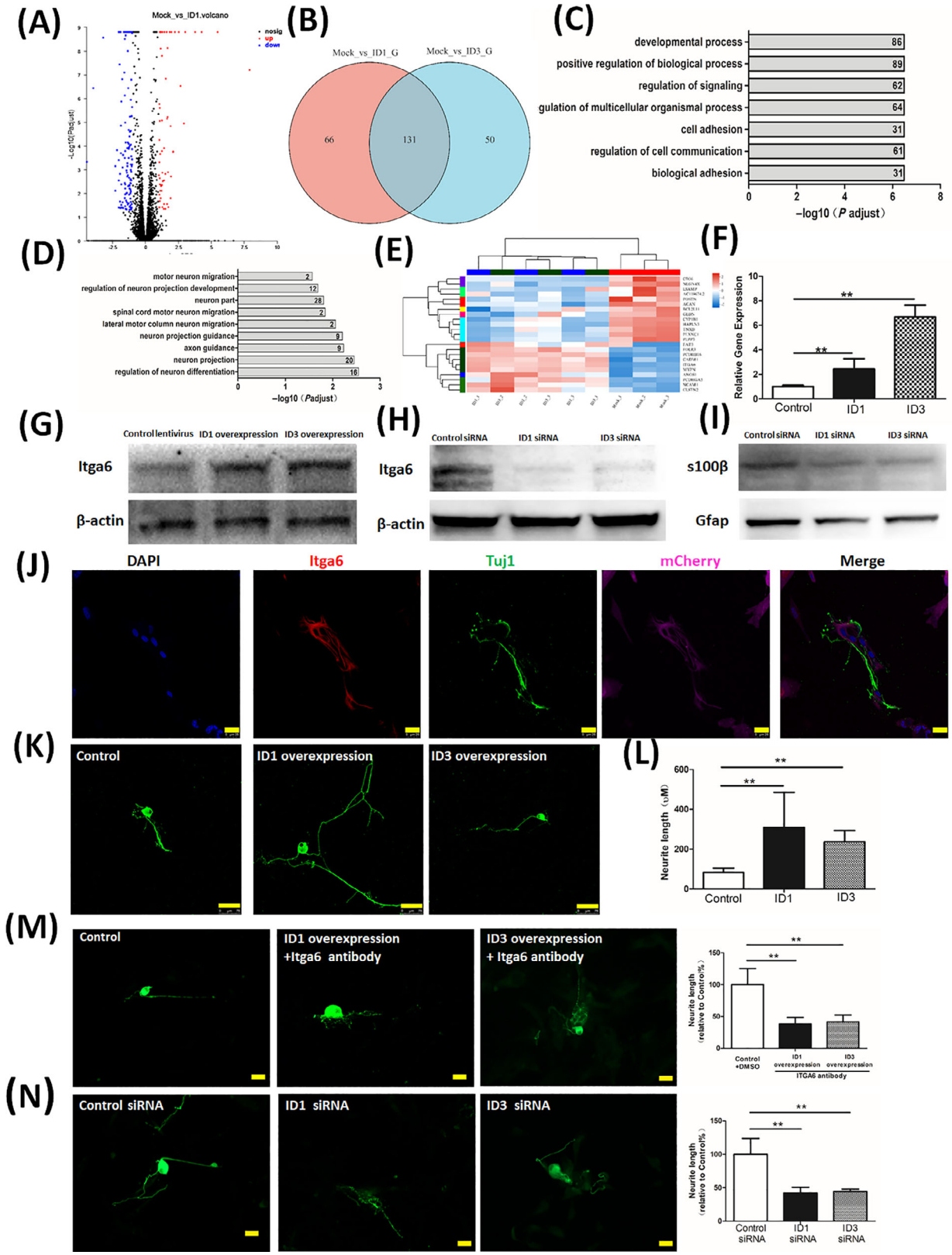

FIGURE 4 ID1/ID3 regulate Itga6 expression in fibroblasts to promote the regeneration of the local nerves. A, Whole-transcript expression profiling of ID1 overexpression human fibroblasts. B, The overlap gene numbers between ID1 and ID3 overexpression human fibroblasts. C, Gene Ontology (GO) terms and GO terms related to "nerve," "neuron," and "axon"; D, overrepresented in upregulated genes of ID1 overexpression human fibroblasts. E, Heatmap showing upregulated genes in ID1 and ID3 overexpression human fibroblasts. Itga6, S100β, Gfap expression following ID1/ID3 transduction (F, G) or knockdown (H, I) in mouse skin fibroblasts. (H) and (I) share the same loading control β-actin as shown in (H). J, Detecting the contact of Itga6 from mCherry labeled mouse skin fibroblasts with rat DRG neurons. The neurite lengths in cocultured DRG neurons and ID1/ID3-overexpressing mouse fibroblasts (K, L) (n = 10), or ID1/ID3-overexpressing mouse fibroblasts with Itga6 antibody (M) (n = 10), or ID1/ID3 knockdown mouse fibroblasts (N) (n = 10). Scale bar = 25 μm in (J, M, N) and 75 μm in (K). Data are mean ± SD. $^{**}P < .01$. DRG, dorsal root ganglion

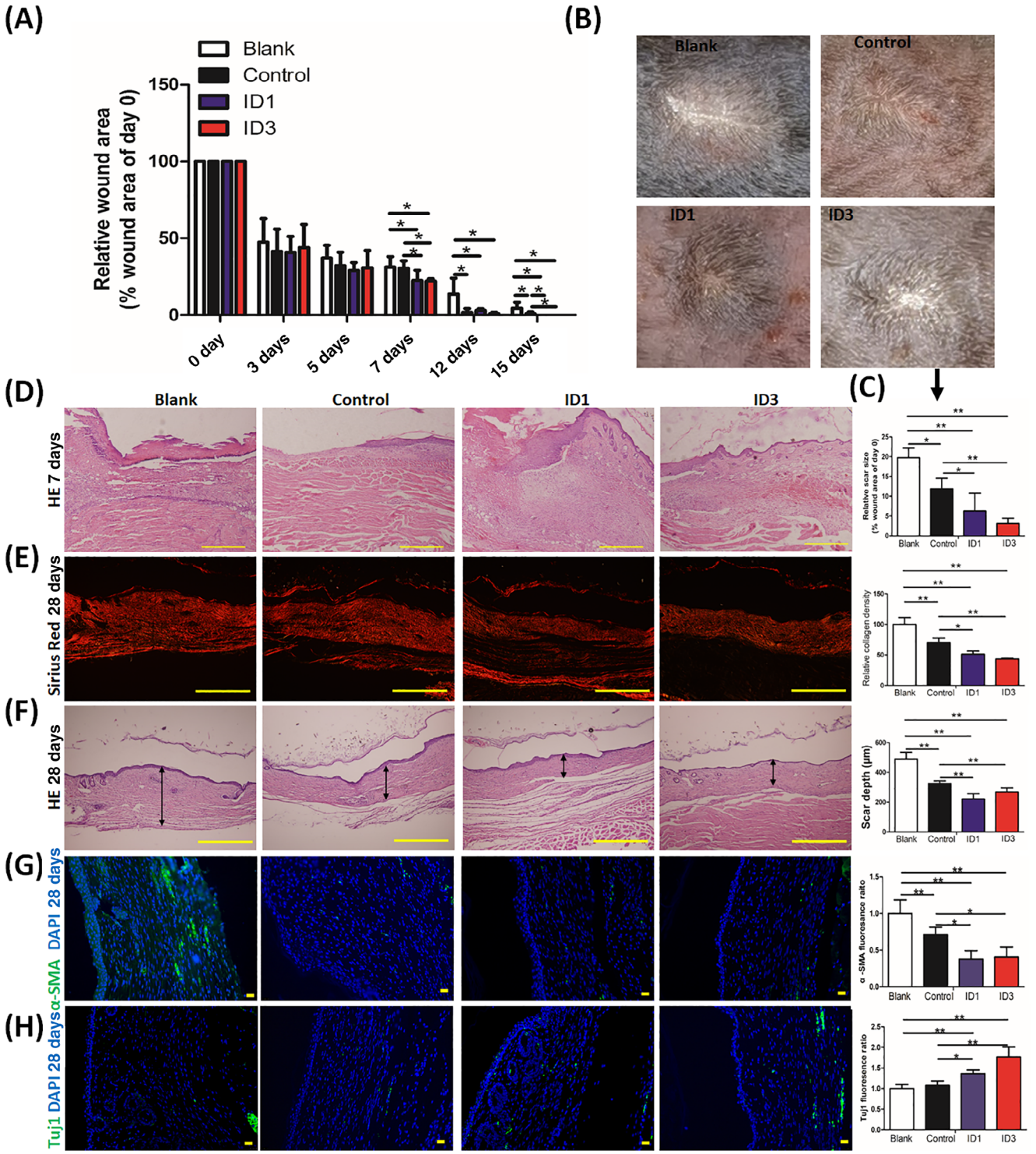

FIGURE 5 ID1/ID3-overexpressing mouse fibroblasts promote the regeneration of the local nerves and wound healing. A, Quantitative analysis of skin wound healing rate in mice topically transplanted ID1/ ID3 overexpression mouse fibroblasts (n = 6). Images and quantitative analysis of, B,C, the scar area, D, granulation tissues, E, ECM deposition, F, scar depth, G, expression of α-SMA, and, H, nerve regeneration (Tuj1 staining) in the wound tissues with topically injecting of ID1/ID3-overexpressing mouse fibroblasts (n = 3). Scale bar = 500 μm in (C, D, E) and 25 μm in (F, G). Data are mean ± SD. $^{**}P < .01$. α-SMA, α-smooth muscle actin; ECM, extracellular matrix

exhibited more tight contact with the nerve fibers than control fibroblasts (Figure 6E). Further costaining results showed many more S100β and Gfap positive ID1/ID3-overexpressing fibroblasts (Figure S10A,B). Moreover, the gross images of innervated gastrocnemius muscles (Figure 6G) and quantification of RGMW among different groups (Figure 6H) illustrated the promotion of regenerated

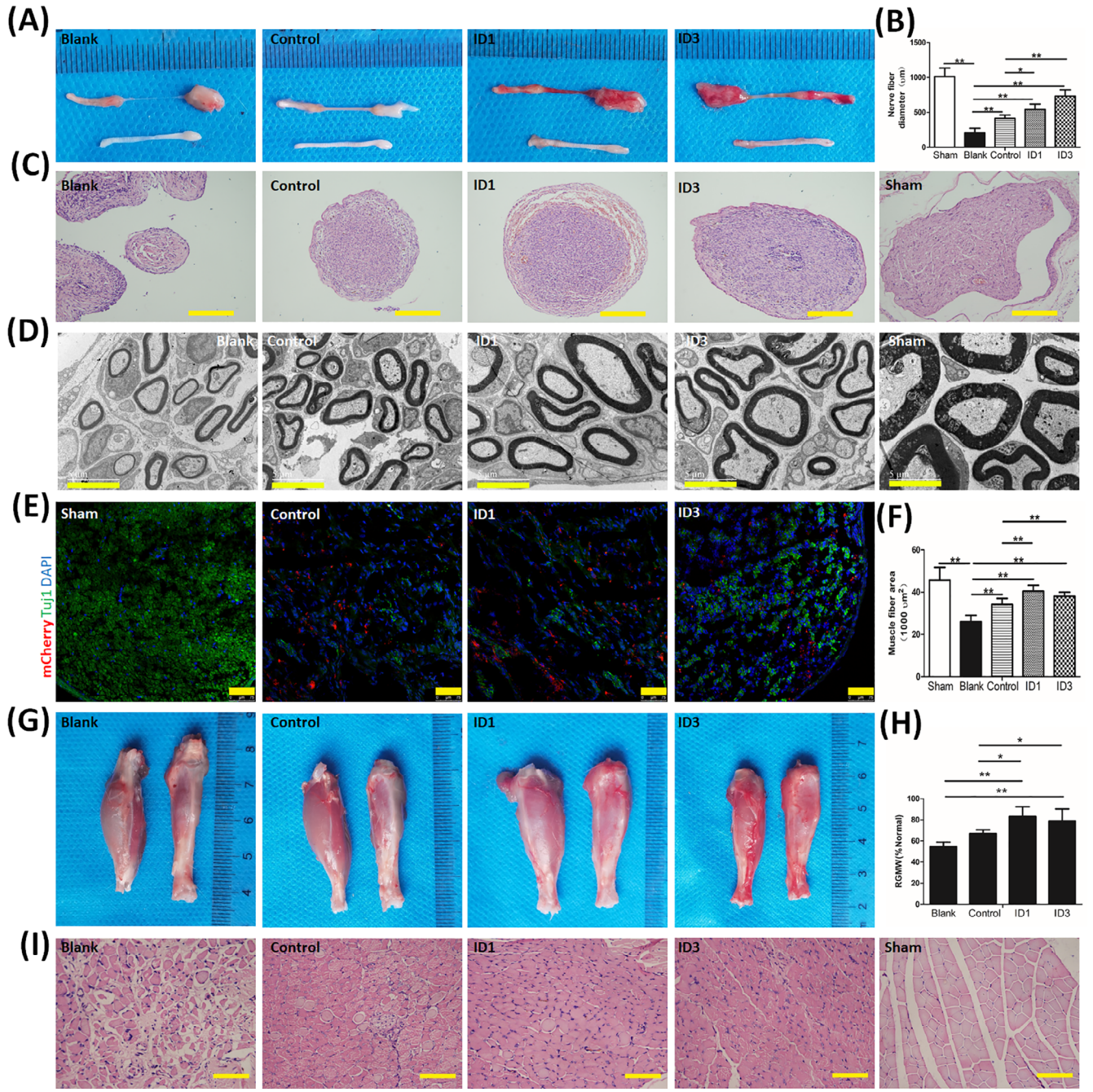

FIGURE 6 ID1/ID3-overexpressing mouse fibroblasts promote the regeneration of the transected sciatic nerves. A, Images of gross morphology, B, quantitative analysis of diameter, C, HE images, and, D, TEM images of regenerated rat sciatic nerve 6 weeks following injury in different treatment groups. E, Immmunostaining the tuj1 and the mCherry labeled control or ID1/ID3 transplanted fibroblasts in the regenerated rat sciatic nerves. F, Quantitative analysis and, I, images of the injured nerve innervation gastrocnemius muscle fiber cross-section area in different treatment groups. G, Images of gross morphology of gastrocnemius muscle and, H, quantitative analysis of relative gastrocnemius muscle weight (RGMW) in different treatment groups (n = 6). Scale bar = 200 μm in (C), 5 μm in (D), 75 μm in (E), and 100 μm in (I). Data are mean ± SD. $^{**}P < .01$. HE, hematoxylin–eosin; TEM, transmission electron microscopy

nerves by ID1/ID3-overexpressing fibroblast transplantation. The cross-sectional area of muscle fibers in the groups (Figure 6F,I) further confirmed more successful reinnervation and less muscular atrophy in ID1/ID3-overexpressing fibroblast transplantation groups.

4 | DISCUSSION

Skin is a highly innervated organ with different types of sensory nerve endings throughout the subcutaneous tissue, dermis and epidermis, and autonomic nerve fibers just as far as dermis.[3] According to

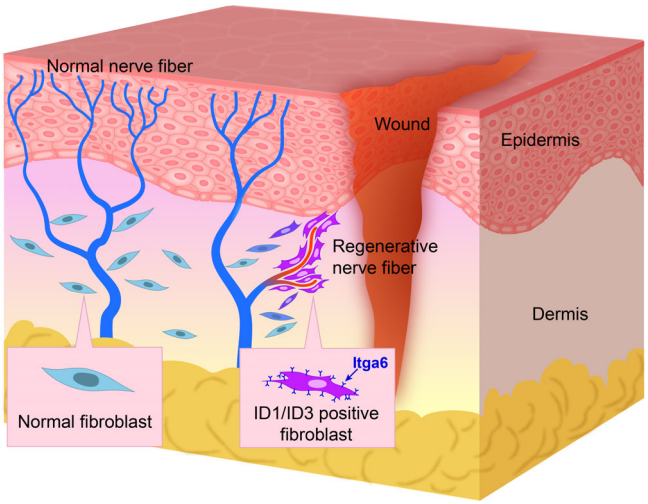

FIGURE 7 Schematic illustration of ID1/ID3 mediates the contribution of skin fibroblasts to local nerve regeneration through Itga6 in wound repair. Following wounding, skin fibroblasts were upregulated ID1/ID3, which induced the increased expression of Itag6. The ID1/ID3 positive fibroblasts were shown to closely contact with the regenerated nerve fibers and contribute to the regrowth of the axon by Itga6

previous studies, skin innervation has been shown to play very important roles in wound healing. Our results also indicated improved skin nerve regeneration after transplanting ID1/ID3-overexpressing fibroblasts, which was accompanied by accelerated and higher quality wound healing. Denervated wounds healed more slowly and even failed to heal.[24] Surgical resection of the nerves to denervate the skin in rats or rabbits impeded wound healing and wound contraction. 6-hydroxydopamine induced skin sympathetic denervation and decreased re-epithelialization and sensory denervation in capsaicin-delayed skin wound healing.[4] The mechanism of skin innervation to improve wound healing is due to the neurotransmitters and neuropeptides produced by both autonomic and sensory cutaneous nerves,[6,7] which take part in multiple phases of the wound healing response; for instance, neuropeptides have been shown to be responsible for inflammatory cell chemotaxis in the inflammation phase.[24] Thus, it is vital to enhance local nerve regeneration for effective wound healing. Although some treatments, including transplantation of Schwann cells,[9] skin stem cells,[25] electrical stimulation,[26] or even mechanical tension,[27] have been shown to improve local nerve regeneration, more effective strategies are still urgently needed. It is particularly important to explore the underlying mechanisms of local axon regrowth further.

The role of fibroblasts, the dominant mesenchymal cell type in the skin, in local nerve regeneration is still unclear. Transplantation of skin fibroblasts has been shown to improve nerve regeneration,[28,29] which is also supported by the results of this study. According to previous studies, fibroblasts were shown to sort the Schwann cells at the injured site and promote their migration to guide axon sprouting.[13] Moreover, fibroblasts have been shown to secret abundant ECM molecules at nerve injury sites to enhance axon regeneration.[12] Whether fibroblasts accelerate nerve regeneration by directly contacting the injured nerve and guiding axon regrowth is not known. Our in vitro and in vivo results indicated that skin fibroblasts were activated to achieve tight contact with the injured nerves and guide axon extension. In addition, transplantation of ID1/ID3-overexpressing skin fibroblasts could also improve regeneration of the sciatic nerves, which exhibited increased diameter of regenerated nerves and myelin sheath thickness. Those morphology of regenerative nerve structures correlated with decreased muscular atrophy and increased SFI. These results showed therapeutic potential for the treatment of peripheral nerve disorders.

The ID family is a group of evolutionarily conserved proteins. Four ID proteins, that is, ID1 to ID4, are found in mammals. ID protein expression is broad but differential in diverse types of cells. Moreover, ID proteins have overlapping expression patterns and share similar biochemical behaviors.[22,23] Previously, ID proteins were shown to be capable of promoting cell-cycle progression, accelerating cell migration, inhibiting differentiation of different types of progenitors, and decreasing cellular senescence,[30] which suggests they play significant roles in neurogenesis,[31-33] stem cell maintenance,[34-37] angiogenesis,[22,38] organ development,[31] tumorigenesis and metastasis,[23,39] and energy metabolism.[40] Moreover, ID proteins have been shown to enhance cell reprogramming. Hayashi's study indicated that ID proteins induced by the bone morphogenetic protein-SMAD signaling pathway improved reprogramming efficiency to pluripotency by suppressing p16/INK4A-dependent senescence.[41] The transduction of ID3 in mouse embryonic fibroblasts resulted in transdifferentiation of fibroblasts into neural stem cell-like cells.[42] In this study, following local in vivo and in vitro wounding, skin fibroblasts were activated to increase expression of ID proteins, which then increased the proliferation and migration of fibroblasts. Skin fibroblasts were reprogrammed into a neural cell-like state. The whole gene expression profile showed that ID proteins also induced upregulated expression of genes related to axon guidance and neuron projection. Further, we identified Itga6 responses to ID protein upregulation as the most abundant differential gene, which was previously reported to mediate Schwann cell interactions with axons and facilitate axonal regeneration.[18] Identification of the ID protein-Itga6 pathway in fibroblast responses to wounding (Figure 7) would shed new light on improvement of peripheral nerve regeneration and potential treatments for chronic wounds.

5 | CONCLUSION

We demonstrated a new role for skin fibroblasts in nerve regeneration following local injury by fibroblasts being reprogrammed into a neural cell-like state and directly contacting and guiding axon regrowth. Furthermore, we clarified the mechanism of fibroblasts contributing to local nerve regeneration through the ID1/ID3-Itga6 pathway, which might hold therapeutic potential in peripheral nerve disorders and peripheral neuropathies with relatively chronic refractory wounds.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Z.C.: conception and design, data analysis and interpretation, financial support, manuscript writing; G.S.: provision of study material, collection and assembly of data, data analysis and interpretation; X.T., L.Q., C.Z., X.C.: collection and assembly of data; L.M., P.L.: provision of study material; F.Y., Y.L.: administrative support; Y.W.: conception and design, final approval of manuscript; C.S.: conception and design, financial support, final approval of manuscript.

DATA AVAILABILITY STATEMENT

All data included in this study are available upon reasonable request by contact with the corresponding author.

ORCID

Chunmeng Shi https://orcid.org/0000-0002-8264-738X

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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