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PDGF-loaded microneedles promote tendon healing through p38/cyclin D1 pathway mediated angiogenesis

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ABSTRACT

Tendon injury is one of the most serious orthopedic diseases often leading to disability of patients. Major shortages of tendon healing are due to its multiple comorbidities, uncertainty of therapeutic efficacy and insufficient of angiogenesis. With a deeper understanding of angiogenic mechanism of tendon healing, we investigated an innovative microneedle patch loaded with platelet derived growth factor (PDGF) to achieve a constant systemic administration of PDGF to enhance topical tendon healing. Rat achilles tendon injury model was performed as in vivo animal models. Histological staining showed an enhancement of tendon healing quality, especially angiogenesis. Biomechanical studies demonstrated an increase of tendon stiffness, maximum load and maximum stress with treatment of PDGF-loaded microneedles. Furthermore, MAPK/p38/Cyclin D1 pathway and angiogenesis were found to play an important role in tendon healing process by using a biological high throughput RNA-sequence method and bioinformatic analysis. The high throughput RNA-seq tendon healing results were confirmed by histochemical staining and western blot. These results suggest the novel therapeutic potential of PDGF-loaded microneedle patch in tendon surgery.

1. Introduction

Tendon injury is one of the most common orthopedic diseases. Except for huge economic costs, tendon injuries could largely impact patients' quality of daily life and their ability to achieve occupational, recreational and health goals [1]. The fibrotic nature of tendon healing further aggravates this burden. After injury, the healing of tendon is not the regeneration of natural tendon structure, but the excessive and disordered deposition of extracellular matrix (ECM) through early-stage angiogenesis. The insufficient healing response is usually attributed to the moderately low number of cells and blood vessels in the tendon [2]. The resulting scars provide moderate tissue stability after injury. However, lack of the mechanical integrity of the original tissue leads to the high risk of re-injury [3]. Furthermore, the fibrotic changes of components of ECM after tendon injury are considered to result in the subsequent development of chronic degenerative tendon disease [4]. In many cases, common sprains and strains of tendon can achieve self-healing without surgical intervention. Nevertheless, healing process is usually time-consuming and leads to aberrant formation of scar tissue, which may take years to reshape into more functional and well aligned collagen tissue. Moreover, more serious injuries, such as complete tears of tendons or ligaments, usually require surgery involvement. The success of these surgical treatments varies [5]. Other treatments, such as cellular therapy [6], tissue engineering [7], allogeneic or xenograft technology [8] and injection therapy [9], have been applied to promote tendon healing. The methods have achieved certain results, but they also have their own limitations. So far, lack of biological understanding of tendon healing mechanism hindered the development of effective treatment methods for tendon injury [5].

Platelet derived growth factor (PDGF) is a serum derived growth factor, which is essential for the maturation of multiple cells, such as smooth muscle cells, fibroblasts and glial cells [10]. PDGF-BB dimer is

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the most studied subtype, which is the only subtype that can bind to all three divergent PDGF receptors (PDGFRs) and initiate different signal cascades [11]. PDGF-BB promotes mitosis and angiogenesis, so as to accelerate tendon healing. PDGF-mediated angiogenesis is commonly coupled with other tissue regeneration process. For instance, PDGF secreted by preosteoclasts could induce angiogenesis coupling with osteogenesis through $CD31^{hi}Emcn^{hi}$ vessels formation [12]. Although special types of vessels have not been found in tendon regeneration, it has been revealed that PDGF signaling pathway closely participated in tendon development and regeneration [13]. Except for angiogenesis, role of PDGF in tendon healing is also multifaceted. PDGF can enroll inflammatory cells, such as neutrophils and macrophages, which are responsible for the decomposition and phagocytosis of debris [14]. In addition, PDGF attracts tenocytes and fibroblasts to migrate to the injured part, and subsequently begins to synthesize ECM, including collagen [15]. Previous studies have shown that this process is regulated by Akt pathway [10]. Role of MAPK/p38 pathway in tendon healing still remained undetermined. When PDGF is used as a biotherapy, the timing and dosage significantly affect its efficacy. The endogenous release of PDGF is at the early stage of inflammation and early proliferation [16], so exogenous PDGF-BB should also be administered at the appropriate time and preferably with delivery methods that allow controlled and sustained release. Traditionally delivered systems are inefficient due to the loss of activity, such as loaded into hydrogel [17]or onto fibrous membrane [18,19]. Thus, there remains urgent need to develop a durable PDGF delivery system that can retain activity.

Microneedles are arrays of small needles. Microneedles can penetrate the superficial skin barrier and avoid contacting the important nerves and capillaries in the epidermis, which provides a more efficient and rapid drug delivery system than the existing transdermal drug delivery strategies [20]. This new method combines traditional injection and patch systems. The drug is administered percutaneous while eliminating pain invasively associated with traditional medical methods [21]. Loading macromolecules or protein into microneedle might have advantages due to the benign stability and protection of PDGF.

In this study, PDGF-BB was loaded into microneedles. Using the rat tendon injury model, we confirmed that the new intermittent systemic administration system could promote tendon healing and activate MAPK/p38 pathway mediated angiogenesis, which may be of great significance in this process. Our work may become a new strategy for the treatment of tendon injuries.

2. Method

2.1. Production of microneedle patch

PVA solution was prepared, and mixed with an appropriate amount of PDGF-BB. Then the mixture was sucked into a breathable but impenetrable Teflon mold, and a vacuum pump was located on the back of the mixture. After filling all the micropores, another PVA sheet was then placed and pressed on the mold. Two pieces of glasses were used to fix the sheet in the pinhole. Two freezing-thawing cycles were carried out to enhance the biomechanical properties of the microneedles, which were easily applied to the dermal surface after thorough drying.

2.2. PDGF release kinetic evaluation

Microneedles were placed in a 24 well plate, added 1 ml PBS and incubated at 37 °C. After 1, 2, 4, 8, 12, 24 h, the release solution was collected and the content of PDGF in each sample was determined by PDGF ELISA kit.

2.3. Rat tendon injury model constructions

All procedures of the animal study were approved by the ethical institutional review committee of Shanghai Jiao Tong University (SYXK(Hu)2016–0020). Sprague-Dawley rats weighing among 250 and 300 g were selected as animal models. After intraperitoneal injection of pentobarbital anesthesia, the skin at the Achilles tendon was incised and the peritendinous tissue was separated to expose the Achilles tendon. The Achilles tendon was transected transversely and sutured with modified Kessler's. Finally, the skin wounds were sewed and disinfected to protect from infection. Rats were randomly divided into three groups: blank group, MN group and MN-PDGF group. The PDGF microneedle patch was replaced at the center of the rat's back at the fixed time every day. Tendon samples were harvested from euthanized rats 3 weeks after operation.

2.4. Histological Evaluation

The Achilles tendons of rats were transected and fixed in the paraformaldehyde, and then the samples were sliced into 5 μm-thickness sagittal section. The sections were stained with hematoxylin-eosin (HE) and Masson's trichrome staining. The histological scoring system was used to grade the repaired sites [22].

2.5. Immunohistochemistry

In brief, tendon sections were deparaffinized, hydrated, and permeated in a permeable liquid. After blocking endogenous peroxidase and nonspecific sites, sections were incubated with anti-p-p38 and anti-col I in a wet box at 4 °C overnight. After incubation with HRP-conjugated secondary antibodies for 1 h, color was developed with DAB solution and counterstained with hematoxylin.

2.6. RNA sequencing and analysis

RNA sequencing (RNA-seq) analysis was carried out by Shanghai XuranBiotechnology Corporation (Shanghai, China). Purification of Poly(A) RNA was extracted from total RNA. Subsequently, poly(A)RNA was converted to double-stranded cDNAs and were then sequenced using the standard procedures according to manufacturers' protocols. The sequencing reads were mapped and calculated the number of reads per kilobase per million mapped reads. Differentially expressed genes (DEGs) were obtained with the inclusion criteria of p value < 0.05 and $|\log$ (Fold Change) $|>2$. Gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed with online tool Sangerbox database (http://sangerbox.com/Tool). The protein-protein interaction network was construct with Cytoscape.

2.7. Promotor prediction

Genomic sequence of Cyclin D was obtained from UCSC Genome Browser (http://genome.ucsc.edu/). Promotor Cyclin D targeted by p38 was predicted by online tool of PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

2.8. Drug-gene interaction

Target drugs of PDGFR were searched by an online drug-gene interaction database (DGIdb, https://dgidb.genome.wustl.edu/) and were construct in a network with PDGFR by Cytoscape.

2.9. Homology modeling and molecular docking of candidate drugs

Alphafold v2.0 (https://alphafold.ebi.ac.uk/) was adopted to build structure of PDGFR as an artificial intelligence prediction tool as conduct in previous studies [23]. The amino acid sequences of PDGFR were obtained from the UniProt-KB database (http://www.uniprot.org/). Local distance difference test (LDDT) score was used for evaluation of the stereochemical quality. After thorough analysis of protein structure, AutoDockTools and PyMol were applied to hydrogenate or delete the crystallographic water and ligands of molecular structure. Related

small-molecules were searched from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov). Autodock Vina with default parameters was applied for eventual molecular docking. Docking affinity was also scored. PyMol was applied to analyze and visualized the optimal docking conformation.

2.10. Immunoinfiltration analysis

Online tool of ImmuCellAI (http://bioinfo.life.hust.edu.cn/ImmuCellAI/) was adopted to analyze and score the distribution of different immune cells.

2.11. Western blot analysis

The tendon tissues of each group were divided into 300 μL of RIPA for 15 min. The total protein concentration was determined using BCA and the same amount of protein sample (20μg/lane) was separated and transferred to polyvinylidene fluoride membrane. The following main antibodies were used: anti-col I (1:1000), anti-p38 (1:1000), anti-p-p38 (1:1000) and anti-CyclinD1 (1:1000). β- Actin (1:10000) was used as internal control. After primary antibody culture, the membrane was cultured in Goat anti rabbit IgG (H & L) horseradish peroxidase conjugate (1:2000) at room temperature for 2 h. The protein was visualized and captured by automatic chemiluminescence imaging analysis system, and quantified by ImageJ software.

2.12. Biomechanical testing

The proximal and distal ends of each Achilles tendon sample were fixed with two dynamometers. Then, the proximal end was stretched at the speed of 20 mm/min until the tendon could no longer extend. The maximum tensile strength of tendon after repair was documented by rheometer.

2.13. Statistical analysis

All values were recorded as mean ± standard deviation (SD). The Students'*t*-test was used to compare between each two groups. Multiple comparisons were made through a one-way ANOVA followed by Fisher's tests. A P-value of <0.05 was considered to represent statistically significant difference [22], $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***.

3. Results

3.1. Characterization of PDGF loaded microneedle

The manufacturing process of PDGF-loaded PVA based microneedle patch was demonstrated in Schematic 1. The diameter of PDGF-loaded microneedle patch was about 10 mm with an array of about 150 needle tips. The microneedle patch was observed under 45x magnifying glass (Fig. 1A and B) and a 40x microscope (Fig. 1C). The representative photos of SEM also demonstrated a uniformed alignment and homogenous shape of tips (Fig. 1D). The microneedle patch was pressed directly to the shaved rat back skin directly without any help of tip materials. No

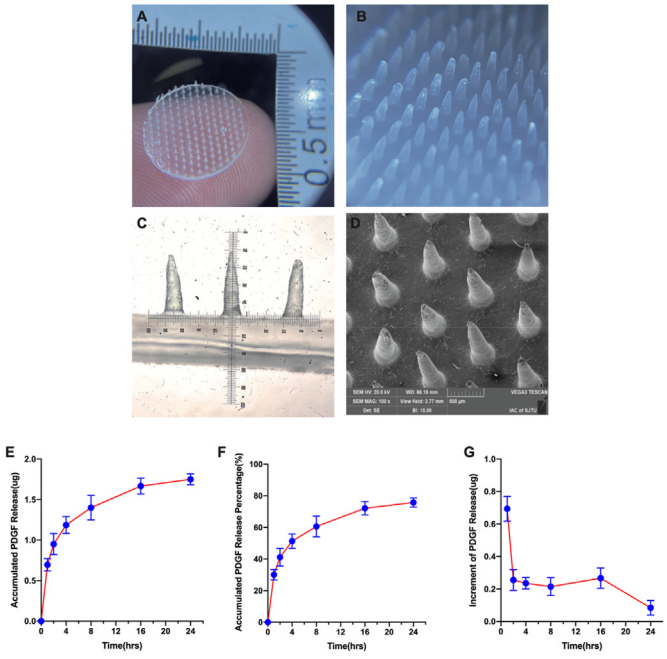
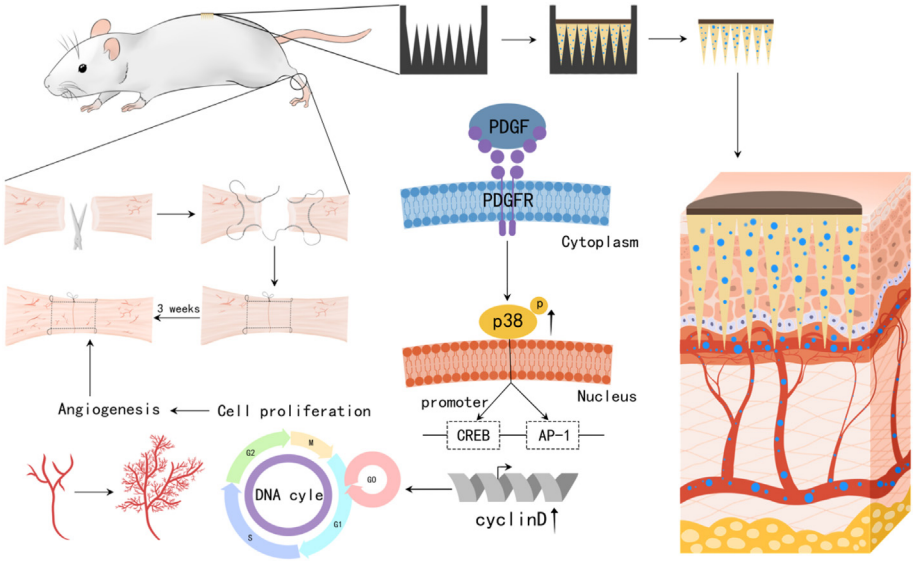


Fig. 1. Characterization of PDGF loaded microneedle. Gross appearance of PDGF-loaded microneedle under 45x magnifying glass (A, B). Microscopic appearance of PDGF-loaded microneedle with 4 × 10 magnifying(C). Topical magnified SEM photos of PDGF-microneedle. (E) Accumulated release of PDGF mass; (F)Accumulated PDGF release of percentage; (G) Increment of PDGF release.



Schematic 1. Graphical abstract of this study. PDGF-loaded PVA based microneedle was fabricated by Teflon mold method. In vivo studies showed an enhancement of tendon healing quality through angiogenesis in rat achilles tendon injury model. Followed up experiments including high-throughput RNA sequence further revealed role of MAPK/p38 pathways in PDGF tendon healing process. After combination of PDGF and PDGFR, MAPK/p38 signaling pathway was activated. Phosphorylation of p38 was entered into nucleus and combined with transcriptional factors. Two predicted promoters of Cyclin D, CREB and AP-1, downstream molecules of p38, were then transactivation and the expression of Cyclin D was upregulated. With the upregulation of Cyclin D, cell proliferation was enhanced leading to angiogenesis and tendon healing enhancement.

obvious undesirable adverse reactions such as redness, bleeding, swelling or increased skin temperature were observed after patch removal. These results indicated the biosafety and the sufficient mechanical strength of these PDGF-loaded microneedle patch.

ELISA kit of PDGF was used to measure the release kinetic characteristics of PDGF-loaded microneedle patch as in Fig. 1. The standard curve of kit was demonstrated in **SI1**. The accumulated PDGF release of mass (Fig. 1E), percentage (Fig. 1F) and the increment of release (Fig. 1G) was calculated and depicted. The release amount of the first 8 h was1.59 ± 0.11 μg and reached about 90% of the total release. And after 24 h, about 80% PDGF has been released. However, due to the PVA enclosure of PDGF, about 20% of the total PDGF could not yet released, which was consistent with previous studies [24].

3.2. Increased ECM deposition and promotion of tendon healing after injury

HE staining and Masson trichrome staining of the injured tendon was used to evaluate qualities of tendon healing. The grading score of tendon healing quality was based on previous study [25] and was demonstrated in **SI 2** in detailed. The enlarged figures shown in Fig. 2A and B indicated that MN-PDGF group had a better tendon healing results with more nascent collagen tissue, lowest histological score including cellularity (Fig. 2C), angiogenesis (Fig. 2D), paralleled fiber (Fig. 2E) and fiber diameter (Fig. 2F), when comparing to the MN group and control group. In particular, less inflammation monocytes infiltration was observed and more angiogenesis vessels lumen was observed in PDGF-MN groups. This result indicated the benign angiogenesis instead of the inflammatory angiogenesis as the post-injury response. No significance of difference existed between pure microneedle group and the control group.

Furthermore, results of immunochemical staining of Collagen Type I were consistent with the Masson results as shown in Fig. 9A. Positive rate of Collagen Type I was 18% in PDGF-MN group, has a significant statistical difference with control group (16%) and MN group (15%). These results indicate PDGF-MN patches could improve tendon healing with better extracellular matrix deposition and benign healing microenvironment.

3.3. Enhancement of biochemical properties

Healing tendon specimens were obtained at 3 weeks for biochemical test, including maximum load, stiffness and maximum stress. The performance of the biomechanical test was best in tendons of PDGF groups in general. As for the maximum load, PDGF-MN treated tendon were almost more than double that of the other two groups. No statistical difference existed between control group and MN group. As for the stiffness, tendons from PDGF-MN groups were 140.6% higher than that of the control groups, and 158.9% of the MN group. As for the maximum stress, tendons of PDGF-MN group can endure 2.1 MPa strength tensile. The result of PDGF-MN group was almost double of the MN group, and was 160.8% of the control group. Thus, the PDGF-MN group has an obvious improvement of the biochemical properties of the healing tendons as shown in Fig. 3.

3.4. Bioinformatic analysis

3.4.1. Screening out the differentially expressed genes (DEGs) of PDGF-MN group and MN group

Benefit from high throughput RNA sequence technique, we further investigated the biological mechanism of PDGF in tendon healing. As

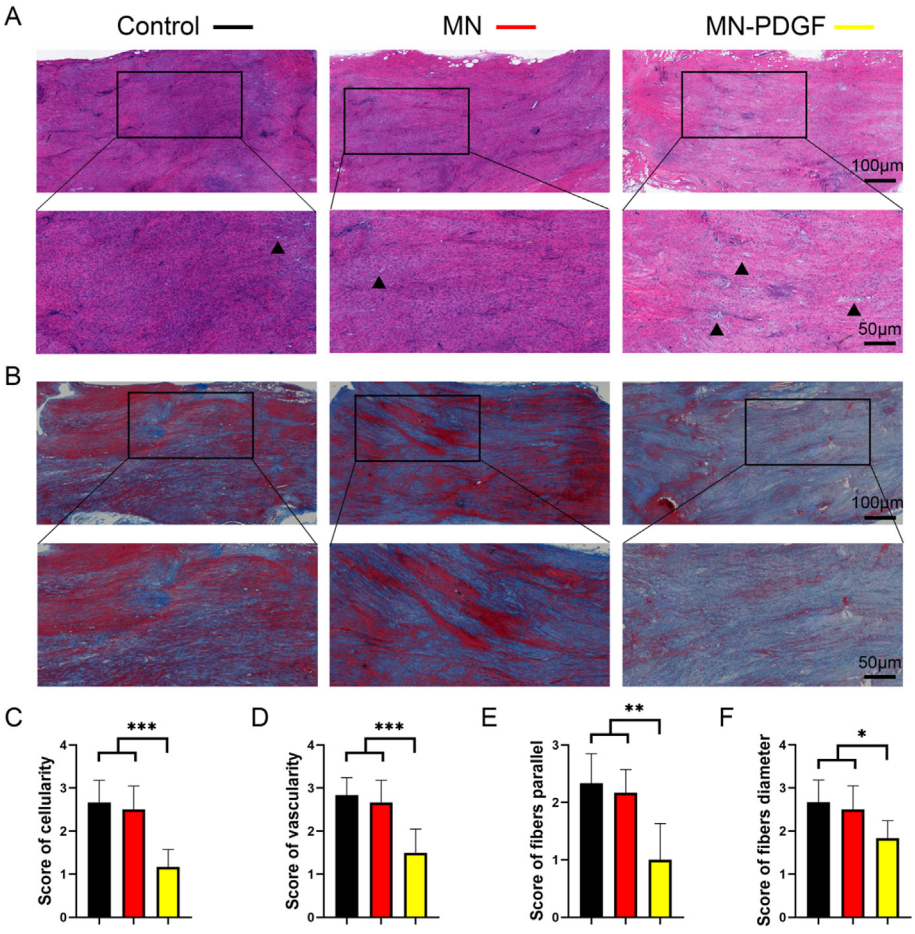


Fig. 2. Histological Evaluation of tendon healing. (A) HE staining and enlarged pictures of the tendon healing areas from control, microneedle and PDGF-loaded microneedle groups. Arrows in the HE figures demonstrated the microvascular structures. (B) Masson staining and enlarged pictures of tendon healing areas. Quantitative analysis of histology score of three groups based on HE and masson staining, including cellularity(C), vascularity(D), fibers parallel(E), fibers diameter(F). ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$.

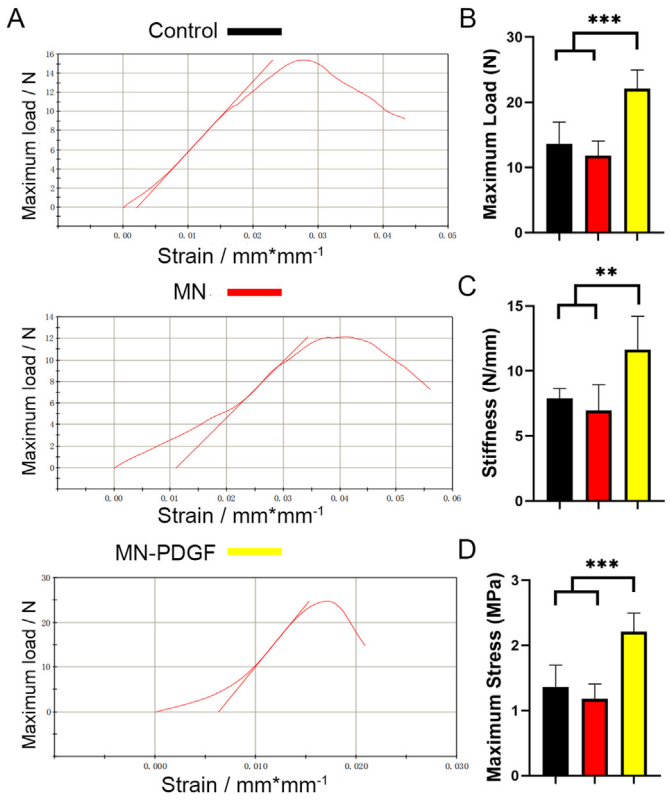


Fig. 3. Biomechanical Test. (A) The maximum load and strain curve of 3 different groups. Quantitative analysis of maximum load(B), stiffness(C) and maximum stress(D). ***, $p < 0.001$; **, $p < 0.01$.

shown in Fig. 4, the expression level of genes in tendons from PDGF-MN treated groups were shown in the heat map. Most of the genes related to tenogenesis were up-regulated, including collagen Type I and Scx. Pro-angiogenic factors of VEGF, HIF-1a, PDGF were also upregulated. Volcano plots were further performed to identify the upregulated and downregulated genes based on the criteria of p value < 0.05 and $|\log$ (Fold Change) $|>2$. Representative genes were marked on the plot with red characters, including MAPK11(p38), PDGFB, PDGFRb, and Jak3.

3.4.2. *Functional annotation and pathway enrichment analysis of DEGs*

After identification of DEGs in the treated groups, GO analysis with functional annotation and KEGG analysis of pathway enrichment were conduct to further investigate the potential biological mechanism as shown in Fig. 5. As for GO analysis, the results mainly enriched from 3 aspects, including biological process (BP), cellular component (CC) and molecular function (MF). As for BP, DEGs were mainly enriched in cell differentiation, cell component organization, cellular developmental process; As for CC, DEGs were mainly participate in cell projection and neuron projection; As for MF, DEGs were mainly involved in protein binding and ion-related channel activity regulation. When it comes to KEGG analysis, DEGs were found to mainly enrich in insulin secretion and MAPK signaling pathway.

3.4.3. *Activation of MAPK/P38 pathway in PPI network*

Benefit from powerful bioinformatics, we construct protein-protein interaction network of DEGs and try to find out the most associated pathway in PDGF mediated tendon healing. As demonstrated in Fig. 6, PPI network of DEGs have made a prediction of the possible involved pathways, including MAPK11(p38), JAK3, PDGFRB, COLA1 and COL1A2. These results indicated the MAPK/p38 pathway was one of the most related signaling pathway in PDGF-PDGFRb mediated tendon healing.

3.4.4. *Predicted regulatory functions of p38 on cyclin D*

With the promotor sequence of Cyclin D, PROMO database has given the prediction of p38 targets of cyclin D. Two of the transcription factors targeted by p38 were cAMP response element-binding protein (CREB) and activator protein-1 (AP-1), marked in red in Fig. 6.

3.4.5. *Homology modeling and molecular docking*

With the help of online tool Drug-Gene Interaction Database, target drugs focused on PDGFR were predicted as Fig. 7A. Although DGIdb was based on FDA drug bank, however, most of drugs were applied in tumor

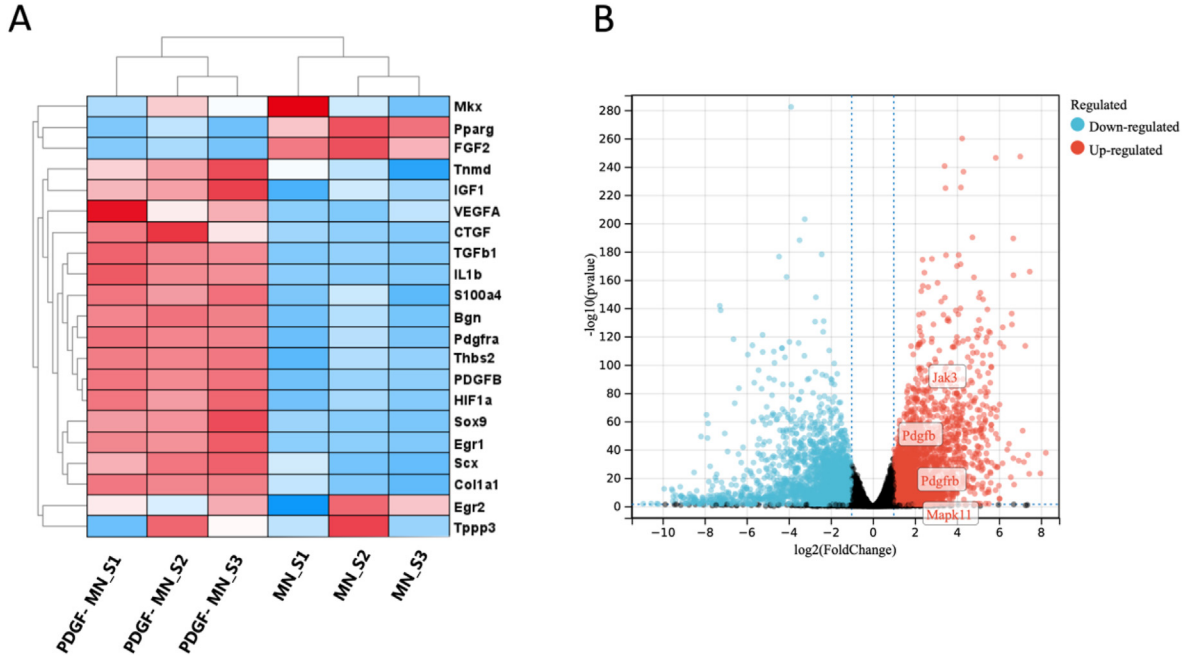


Fig. 4. DEGs of PDGF-treated in tendon healing group. (A) Heatmaps of RNA-sequence results. (B) Volcano plots of candidate DEGs in the microarray datasets based on the screen criteria. PDGFB, PDGFRb, and Jak3 were upregulated and were marked with red characters.

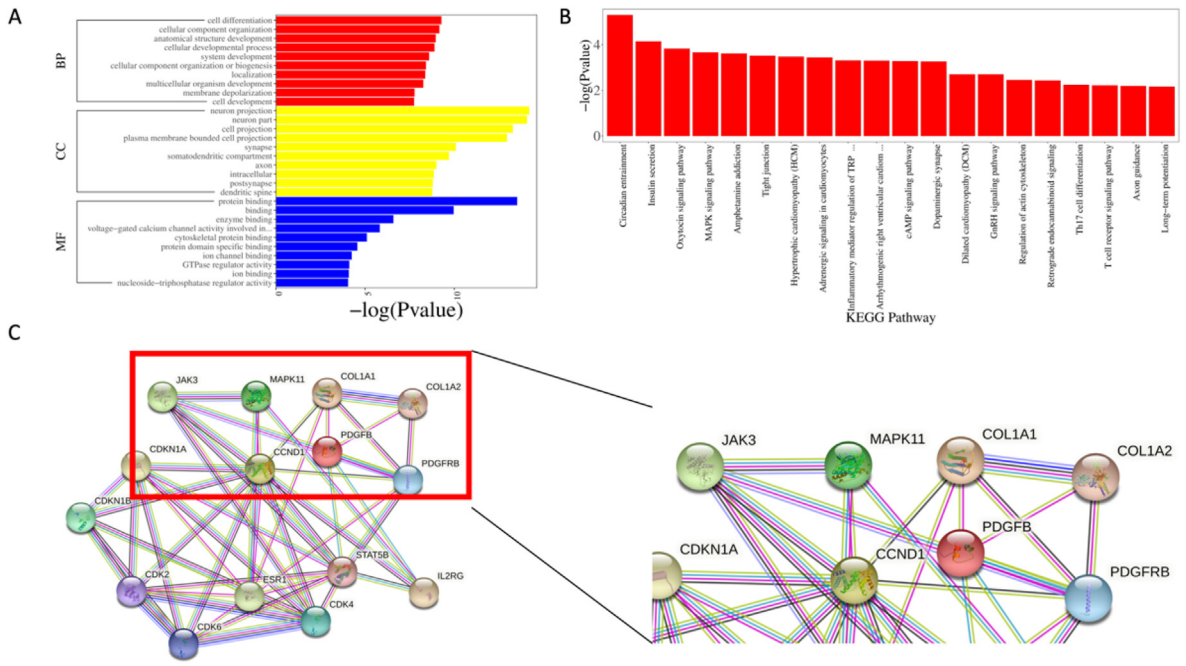


Fig. 5. Functional annotation and pathway enrichment analysis of DEGs. (A) Functional annotation of GO analysis showed the enrichment of DEGs from three aspects, biological process (BP) in red, cellular component (CC) in yellow and molecular function (MF) in blue. (B) KEGG pathway enrichment results. (C) PPI network of DEGs and module analysis. The enlarged parts shown that MAPK11(p38), JAK3, PDGFRB, COLA1 and COL1A2 had most of the modules and clusters.

Fig. 6. Promoter analysis of Cyclin D. (A)The prediction of transcriptional factors of Cyclin D through PROMO. (B)One of the transcription factors targeted by p38 was cAMP response element-binding protein (CREB). (C) Another of the transcription factors targeted by p38 was activator protein-1 (AP-1).

field. Thus, our results were mainly enriched on monoclonal antibody of tumor targeted drugs.

With the considerable achievements of computational biology, drug-gene database was founded, crystal structure of target protein could be predicted. The crystal structure of PDGFR was construct by AlphaFold 2.0 as in Fig. 7B. Local distance difference test (LDDT) score of PDGFR was also demonstrated to evaluate of the stereochemical quality of PDGFR in Fig. 7C.

Furthermore, homology model of PDGFR and small molecules from Pubchem Database was used to screen out the highest affinity drugs to PDGFR. The results indicated that sotrastaurin has the strongest binding affinity towards PDGFR (6.8–8.7 kcal/mol) as shown in Fig. 7D. The binding sites between sotrastaurin and PDGFR were at GLU 835, LYS 840, GLU 681 and HIS 961 through hydrogen bonds as enlarged in Fig. 7E. The absolute value of affinity between predicted small molecules and PDGFR was also exhibited in Fig. 7F.

3.5. Validation of role of MAPK/p38pathway in tendon healing

Based on the above-mentioned prediction of MAPK/p38 pathway engaged in PDGF mediated process, validation experiments were taken to support our hypothesis. Western blot results of collagen type 1, p-p38,

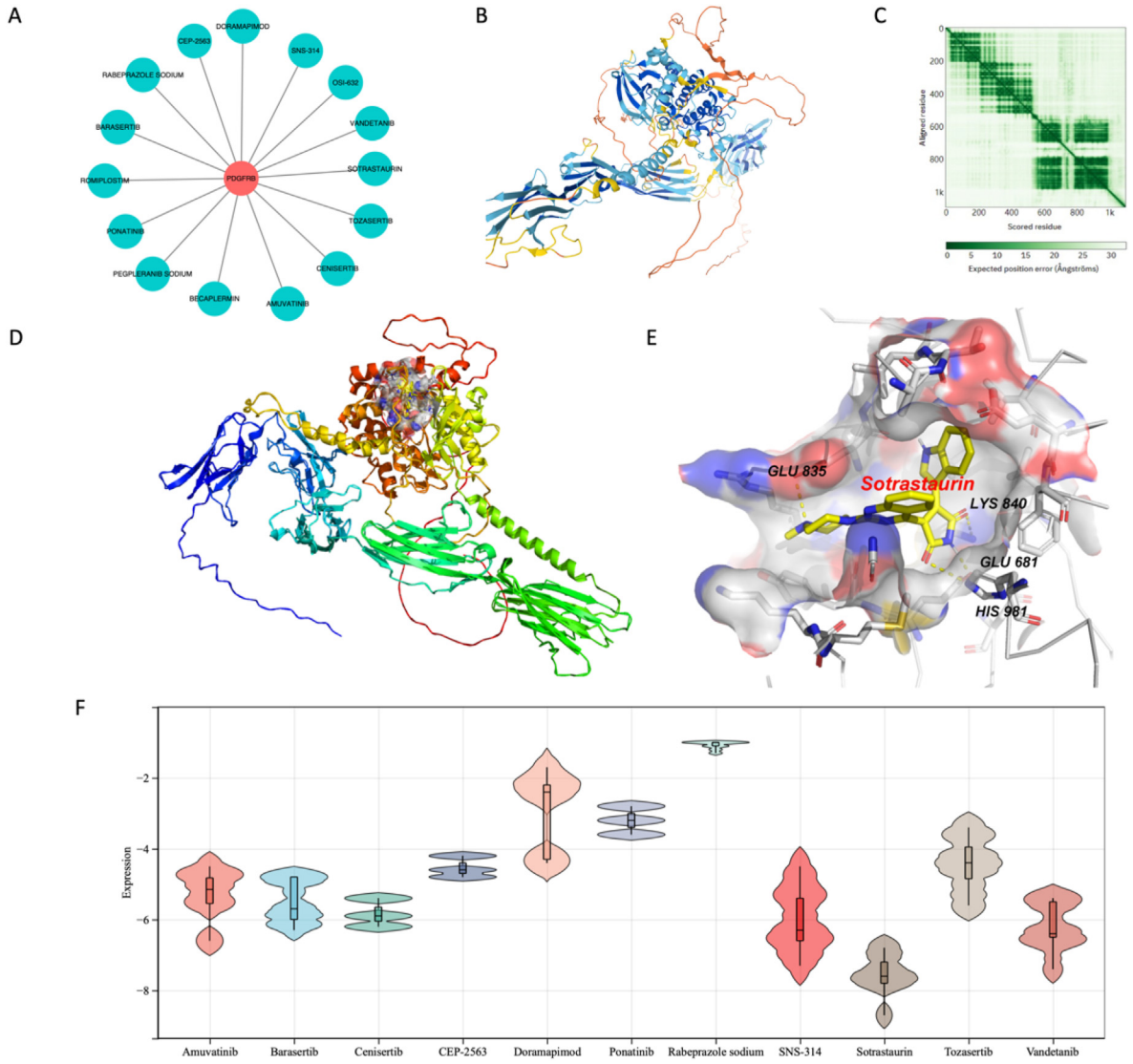


Fig. 7. Drug-gene interplay, homology modeling and molecular docking. (A) Drug-gene interplay of PDGFR. (B) crystal structure prediction of PDGFR by AlphaFold 2.0. (C) Local distance difference test (LDDT) score of PDGFR. (D) Molecular docking of sotrastaurin(E) has the strongest affinity with PDGFR at GLU 835, LYS 840, GLU 681and HIS 961 through hydrogen bonds. (F) The absolute value of affinity between predicted small molecules and PDGFR was exhibited.

Cyclin D and β-actin showed an upregulated expression level as in Fig. 8, suggested a better extracellular matrix deposition and an enhancement of cellular proliferation.

The trend in immunochemical staining of phosphorylated p38 was consistent with collagen type I staining. PDGF-MN group has the most positive ratio of p-p38 comparing to the MN and control group as shown in Fig. 9. These results verified the hypothesis that PDGF might activate p38phosphorylation in PDGF mediated tendon healing.

4. Discussion

In our study, we investigated a PDGF controlled release microneedle patch to promote tendon healing process through MAPK/p38 pathway mediated angiogenesis in a rat Achilles tendon injury model.

Tendon healing process was long been bothered by several complications, such as peritendinous adhesion, tendinopathy or malunion of tendon [26]. Acceleration of this process could promote the quality of healing and the early start of rehabilitation. The extrinsic quality of tendon healing after injury is determined by early vascularization in the healing area, then leading to the proliferation of peritendinous related cells and aligned deposition of extracellular matrix [27]. PDGF-BB has long been recognized as an important mitogen for mesenchymal tissue naturally [13]. Thus, enhancement of the proliferation of tendinous cells through exogenous PDGFBB might realize the high quality of tendon healing.

In this case, an efficient, accurate dosing, controlled released method was encouraged to design for tendon healing process [28]. For the first time, PDGF was loaded into microneedle patch. Previous studies have reported several PDGF delivered system, including gelatin hydrogel [17], heparin collagen fiber [29] and polyester urethanetube [30], etc. Nevertheless, these methods indeed obtained satisfied results to some extent, the easy-degradability of bioactivity of biomacromolecules has not been taken into account. Loading PDGF to PVA based microneedle patch would not lose the bioactivity of PDGF. Microneedle patch system has a benign stability of macromolecules, such as insulin, due to poor intermolecular affinity and low polymer water solubility [31]. Another innovative application of microneedle loaded PDGF was the thermostability. Instead of keeping the PDGF loaded biomaterials in freezing environment, the PDGF-MN could be transported and stored in room temperature. Besides, the microneedle patch was also advantaged by its convenient use and change, no topologically irritation of other biomaterials and avoid of pulse release of the loaded drug [32]. The PDGF

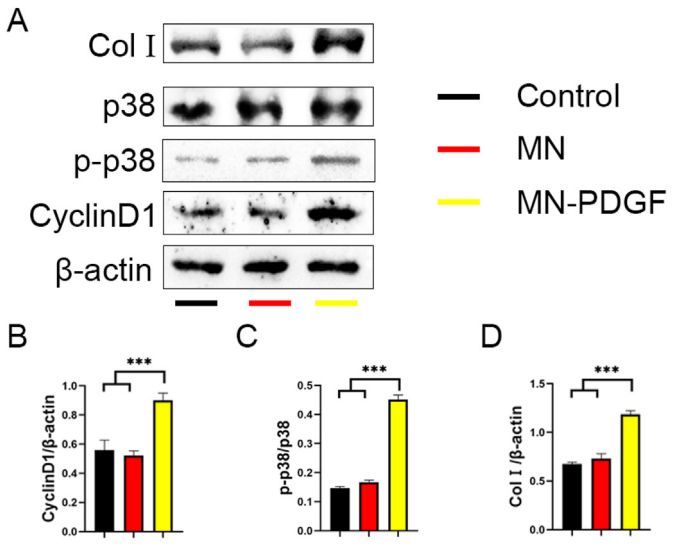


Fig. 8. Western blot of MAPK/p38 pathways proteins. (A)Western blots results of Collagen Type 1, p38, p-p38, cyclin D and b-actin. Quantitative analysis of protein relative expression level of cyclinD (B), p-p38 (C), and Collagen Type 1 (D). ***, $p < 0.001$.

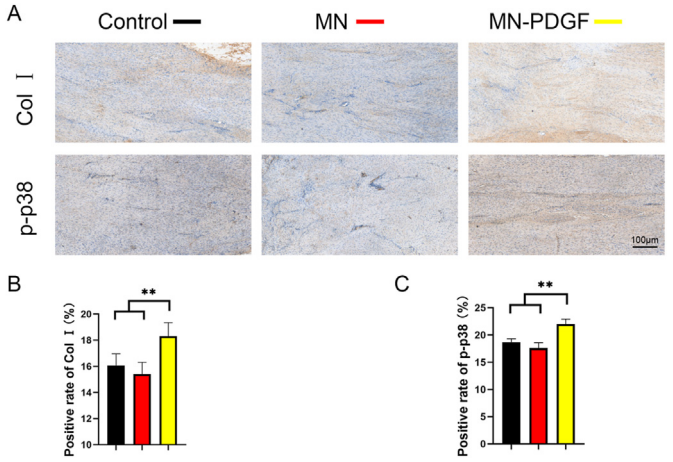


Fig. 9. Immunochemical staining of repaired tendons. Representative immuno-histochemical staining for COL1and p-p38 (A) in repaired tendon sections from different groups. Quantitative analysis of positive ratio of Collagen Type 1(B), p-p38(C). *, $p < 0.05$; **, $p < 0.01$.

transdermal administrative route via a theoretically noninvasive pattern without any safety concern comparing with conventional injection route. This gave the microneedle patch a more competitive opportunity for translation, such as the preclinical studies.

In tendon injury model, early tissue injury process always accompanied by hemostasis and inflammation, while PDGF was largely derived from platelet, macrophage, monocytes and etc [11]. Hence, in early injury process, endogenous PDGF has a relatively high topological concentration due to the post injury response [33]. Exogenous PDGF might reconstruct the functional vascularity and perfusion of the healing area [34]. Early-stage enhancement of angiogenesis by exogenous PDGF might also accelerate tendon healing by promoting extrinsic tendon healing. One of the vital properties on tendon healing is the biomechanical features. Early-stage microvascular capillaries induced by PDGF probably leading to the proliferation of myofibroblasts and collagen deposition. Studies have shown that myofibroblast proliferation of avascular tissue treated by PDGF [35] was probable the major factor to increase mechanical property [36]. Conventional drug delivery system like direct inject or nanoparticle release might have a pulse increase of PDGF. However, immediately high concentration of PDGF might leads to the enormously increase of expression of VEGF. The abnormal angiogenesis could be harmful to the tendon healing process, especially at later stage [37]. In our drug release tests, PDGF was steadily released from microneedle patch in the first hours. Subsequent release was also steadily controlled, without any sudden release. Microneedle patch loaded with certain concentration of PDGF could be changed conveniently after thorough release to achieve the anticipated plasma concentration. Moreover, in the future, the microneedle patch might load multiple growth factors as a cocktail strategy for tissue engineering.

Besides the intermittent administrative method for tendon healing, the biological mechanism of PDGF was also interested to discuss. PDGF has long been recognized as a reparative and regenerative growth factor in many biological processes. PDGF has several functions, including mitogenic effect, promote angiogenesis, induction of stem cells tenogenesis, and extracellular matrix deposition [38]. From the perspective of angiogenesis, in depth studies have shown that endogenous PDGF has been related to inflammatory angiogenesis [39]. Moreover, studies have reported that PDGF-BB signaling mediated cellular secretion of high mobility group box 1 (HMGB1) in mechanical stress treated vascular smooth cells, explaining vascular dysfunction in stressed vasculature [33]. Interestingly, exogenous recombinant PDGF could enhance functional vascularity and sufficient tissue perfusion to consequently promote tissue outcome [34]. As stated in our manuscript, the angiogenic enhancement was related with less macrophages and cellularity score. Besides, PDGF could elevate expression level of VEGF and enhance cell proliferation thus result in angiogenesis [37], as shown in our RNA-seq results. PDGF can also promote tissue regeneration through angiogenesis by p-PI3K, p-Akt, and p-eNOS [40]. Few studies have reported the tenogenic effect of PDGF in tendon tissue. PDGF has been demonstrated to induce tenogenic differentiation of adipose derived stem cells under the combined action of GDF6 [41]. Furthermore, recent studies have revealed that TPPP3 positive cells were essential to PDGF pathways and PDGF related ECM deposition, even tendon fibrosis [13].

PDGF exerts effect by combination with PDGFR and activation of the downstream signaling cascades. Multiple ECM anabolic signaling pathways, including Akt [42], JNK, Erk2 and MAPK, have been demonstrated to be initiated under exogenous PDGF [11]. However, the role of MAPK/p38 activated by PDGF in regulated biological process still remained paradoxically in debate. Conventionally, p38 is a tumor suppressor gene, and respond to various external stressors such as reactive oxygen species and hypoxia thus leading to cell apoptosis [43]. However, facing with growth factors activation, p38 can function as a tumor promoter, involving angiogenesis, cell migration, etc [44]. Zhou et al. reported that nintedanib, a receptor tyrosine kinase inhibitor, including inhibition of PDGF, lead to suppress phosphorylation of p38 [45]. Rieg et al. also found that PDGF-BB/PDGFR regulates the pulmonary vascular tone by the activation of MAPK/p38 or PI3K/AKT/mTOR signaling [46]. Interestingly, it has also been demonstrated that exertion of PDGF activated MAPK in an intervertebral disc degeneration model, but not through phosphorylation of p38 [47]. This might be attributed to the binary equivalent regulatory mechanism of p38 on apoptosis and proliferation. Thus, MAPK/p38 pathway could functioned as a vital downstream pathway of PDGF to regulate cell survival and proliferation metabolism.

As shown in our RNA sequence results, two important pro-angiogenic factor VEGFa and HIF-1α and p-p38 were all upregulated significantly. HIF-1a is a biomarker of hypoxia and angiogenesis, however, long term abnormal angiogenesis of HIF-1a signaling pathway might leading to tendinopathy [48]. HIF-1a activation in tendon healing mainly functioned on neovascularization and stem cell *trans*-differentiation. On one hand, hypoxia proangiogenic environment post tendon injury was

possibly enhanced with PDGF and leading to more angiogenesis at early stage of tendon healing in our study. Previous study has also reported the angiopoietin-like 4 (ANGPTL4) could regulate tendon neo-vascularization through TGF-β and HIF-1α comprise two signaling pathways under cyclic stress [49]. On the other hand, enhanced HIF-1a level could help retain stemness of tendon stem cells through suppress of Smad7 [50]. HIF-1a could also enhance human tendon stem cells self-renewal and differentiation through binding EGR1 promoter [51]. Previous studies have revealed that activation MAPK/p38 pathway results in enhancement of angiogenesis and VEGF secretion [52]. Leelahavanichkula et al. reported that p38α inhibition resulted in remarkable decrease in intra-tumoral blood and lymphatic vessels of head and neck squamous cell carcinoma [53]. Nevertheless, the conventional function of p38 was still worthy of attention as its function in pro-apoptosis. This may due to multiple reasons, such as the differences between post-injury regenerative microenvironment and tumor microenvironment, the sub isoform-dependent mechanisms act on Ras signaling between p38α and p38γ [44].

Phosphorylated p38 might enter into the nucleus of cells to target the downstream transcriptional factors (TF) and promote cell proliferation. CREB and AP-1 are one of two vital TFs targeted by p-p38 [54], existed on promoter of CyclinD1. CyclinD1 is one of the cell cycle related proteins. Upregulated expression level of CyclinD1 often gives hint of cell proliferation. In our study, upregulated protein levels of cyclinD indicated cell proliferation and differentiation, thus enhanced tendon healing.

Based on the target receptor of PDGF, sotrastaurin was screened out to have the highest affinity to PDGFR. Sotrastaurin was a PKC inhibitor has been applied to target melanoma [55]. However, its potential effect on tendon healing should raise worth of attention due to high affinity of PDGFR.

Our study also has several limitations, including lack of concentration gradient, no optimization of PDGF release time and no control group like topologically treated PDGF etc. The pathway inhibitors should be used to verify the involvement of the pathways. Further investigations of large sample size and sufficient drug loading control groups might deepen our understanding of real-time PDGF concentration in extracellular matrix during tendon healing process, and design more efficient PDGF delivery system to promote tendon healing.

5. Conclusions

In short, we have demonstrated that sustainable release of PDGF from microneedle could significantly increase tendon healing with high efficiency in a rat tendon injury model. The microneedle drug delivery system, characterized by easy fabrication, controlled release and accurate dosing, revealed a promising clinical translational future in tendon healing field. The high through-output RNA-seq screening revealed that PDGF/p38/Cyclin D1 pathway was an important regulator of ECM deposition such as angiogenesis during tendon healing. The prediction of drug sotrastaurin might have preclinical translation in clinical practice.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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