Transcription and proteome changes involved in re-innervation muscle following nerve crush in rats

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Abstract
Severe peripheral nerve injury leads to the irreparable disruption of nerve fibers. This leads to disruption of synapses with the designated muscle, which consequently go through progressive atrophy and damage of muscle function. The molecular mechanism that underlies the re-innervation process has yet to be evaluated using proteomics or transcriptomics. In the present study, multi-dimensional data were therefore integrated with transcriptome and proteome profiles in order to investigate the mechanism of re-innervation in muscles. Two simulated nerve injury muscle models in the rat tibial nerve were compared: the nerve was either cut (denervated, DN group) or crushed but with the nerve sheath intact (re-innervated, RN group). The control group had a preserved and intact tibial nerve. At 4 weeks, the RN group showed better tibial nerve function and recovery of muscle atrophy compared to the DN group. As the high expression of Myh3, Postn, Col6a1 and Cfi, the RN group demonstrated superior re-innervation as well. Both differentially expressed genes (DEGs) and proteins (DEPs) were enriched in the peroxisome proliferator-activated receptors (PPARs) signaling pathway, as well as the energy metabolism. This study provides basic information regarding DEGs and DEPs during re-innervation-induced muscle atrophy. Furthermore, the crucial genes and proteins can be detected as possible treatment targets in the future.

Keywords: Re-innervation, Denervation, Peripheral nerve injury, Skeletal muscle atrophy, Transcriptomics, Proteomics

Background
Skeletal muscle denervation is a common disease caused by trauma, inflammation, or disease. It is well-known that denervated muscle undergoes atrophic changes, involving disruption of sarcomere and myofibrilla, and the degree of atrophy increases with the duration of denervation [1]. In order to analyze the gene expression profiles induced by denervated muscle atrophy, microarray analysis has been used in the previous survey, as well as the RNA sequencing (RNA seq) [2]. Several proteomics studies of denervated skeletal muscle have also revealed biomarkers and pathways involved in denervation-induced muscle atrophy [3]. In spite of previous understanding of the mechanism for denervated nerves, the re-innervation response to crushed nerves has not been well characterized.

The effects of axotomy on motoneurons, nerves and muscles implicate various morphological, biochemical and physiological changes. After a nerve is transected, the damage axons will regenerate following their unique pathway, and eventually re-innervate
the muscles. As previously described, nerve transection will redirect regenerated axons to inappropriate pathways [4]. In contrast to axotomy, the experimental technique of crushing maintains the stability of nerve sheaths and basement membranes. Despite the fact that advances have been made in clarifying the mechanisms of axon and muscle regeneration, the underlying mechanism for re-innervation has yet to be evaluated using a proteomic or transcriptomic approach.

Therefore, the current study aims to investigate possible functional mechanisms by identifying differentially expressed mRNA and proteins in re-innervated muscle over a 4-week period following injury. Furthermore, possible treatment targets might be identified by using bioinformatic analysis, including Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The 4-week study period covers the time when neurogenic muscular atrophy is still reversible and nerve re-innervation and myogenic regeneration can still occur [5, 6]. This research brings new insight into molecular mechanism of nerve re-innervation, while identifying specific targets and pathways for the novel treatment of nerve re-innervation.

Results
General observation
Daily observations of animals in each experimental group revealed normal behavior and no systemic or local inflammation or post-operative complications.

Tibial function index (TFI) analysis
TFI analysis revealed that the TFI values decreased after denervation and crush, and repair increasingly with time. The TFI values in the RN and DN group were significantly lower than in the control group at every time point, respectively \( (P<0.001, \text{Fig. 1a}) \). The TFI values in the RN group were significantly higher than in the DN group at every time point \( (P<0.001, \text{Fig. 1a}) \).

Muscle wet weight
The gastrocnemius muscle wet weight was scaled at 4 weeks (Fig. 1b). To deeply know the muscle mass, we compared the ratio of muscle samples between surgical and healthy side. The muscle mass of the control group is 100% retained. By 4 weeks, DN group revealed a poor return of muscle recording only 86.3% ± 7% \( (P<0.05) \). In RN group, it was 96.2% ± 0.6% which was well recovered compared with the DN \( (P<0.05) \).

Differentially expressed genes (DEGs) and proteins (DEPs)
Untargeted transcriptome and proteome analyses of muscles were performed to explore repair mechanisms in the DN and RN groups (Fig. 2a). A total of 567 genes were identified as differentially expressed genes (DEGs) at the transcriptome level, out of which 151 were altered in DN mice relative to control mice, while 441 DEGs were altered through re-innervating (Fig. 2a). Upon comparing the DN group with the RN group, 344 DEGs were found to be significantly up-regulated, while 97 DEGs were significantly down-regulated. Compared with the control group, 19 genes were down-regulated in DN group, while

Fig. 1 RN group achieved a higher TFI value and better protection for the gastrocnemius muscle. a The tibial functional index (TFI) was determined after analysis of the walking trajectory. The higher TFI values represent better functional recovery. #RN group versus control group, *DN group versus control group. Results statistically significant at \( P<0.05 \) (*, #), \( P<0.01 \) (**, ##) and \( P<0.001 \) (***, ###). b The ratio of muscle on the surgical side compared to the healthy side. * Differences were statistically significant at \( P<0.05 \). By 4 weeks, RN group revealed a well recovery of muscle compared with the DN \( (P<0.01) \)
restored through re-innervating. In the same way, 6 genes were up-regulated in DN group and restored through re-innervating. These results were visualized in the hierarchically clustered heatmap (Fig. 2b) and volcano plot (Fig. 2c). Heatmap representation of hierarchical clustering of DEGs that were significantly altered by RN.

At the proteome level, a total of 385 proteins were differentially expressed. The Venn diagram in Fig. 2d shows the number of differentially expressed proteins (DEPs) in all 3 groups. Among the 223 proteins that were significantly affected by RN, 70 were up-regulated and 153 were down-regulated. Compared with control group, 10 proteins were down-regulated in DN group, and then restored through re-innervating, while 69 proteins were up-regulated and restored through re-innervating. The corresponding heatmap and volcano plot are shown in Fig. 2e, f.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways for the Differentially expressed genes (DEGs) and proteins (DEPs)

Pathway enrichment was analyzed against KEGG database to obtain the potential functions of the DEGs and DEPs. As the results shown in Figs. 3a, b, c, d, DEGs and DEPs were all enriched in pathways involving in PPAR signaling pathway, while DEPs were significantly enriched in pathways related to energy metabolism. Among 126 DEPs distributed in the profile 5 of the short time-series expression miner (STEM) analysis (Fig. 3f), 55 DEPs were related to energy metabolism and suggested RN group a positive effect on energy metabolism comparing with DN group.

Analysis combined transcriptome with proteome data

Furthermore, we combined the transcriptome and proteome data to identify genes that were altered in both omics’ layers. The Venn diagram in Fig. 4a shows the number of co-regulated DEGs/DEPs, and diff-regulated DEGs/DEPs between the DN group and control group. In the transcriptome level, 209 DEGs were up-or-down-regulated, while 1344 DEPs were up-or-down-regulated in the proteome level. Combined with transcriptome and proteome data, 53 were co-regulated while 61 were diff-regulated. Compared with RN group, 96 were co-regulated while 71 were diff-regulated (Fig. 4d). Of the 5 genes
were found to be up-regulated in DN group (Fig. 4c, Q3), out of a total of 2534 genes co-regulated (Fig. 3b, Q3). In RN group, 4 genes differed significantly at both the protein and mRNA levels compared with DN group (Fig. 4f, Q3). These included Myh3, Postn, Col6a1, Thbs4, Cfi and so on, which are widely known to be related with muscle development, energy metabolism and neuronal differentiation.

Discussion

Several studies support the notion that longer de-innervated periods cause reduced re-innervation and ultimately reduced the recovery of muscle function [7]. Significantly, the re-innervation of peripheral nerves is known to influence the recovery of denervated muscle [8]. These observations highlight the need to re-innervate muscles during the early recovery and before unrecoverable neural impairment occurs. Hence, there is considerable interest in identifying the molecular mechanisms of repair using nerve injury models so as to avoid irreversible outcomes post-operatively. Gene expression studies performed with various muscle injury models have reported that certain genes may be critical for achieving a good clinical result [9, 10]. To date, however, the importance of nerve integrity in the repair and recovery of nerve injury is not known. In the present work, the tibial nerve was crushed near the sciatic nerve branch, conducting a re-innervated model and studying gastrocnemius muscle specifically. Moreover, we conducted transcriptomic and proteomic analyses of muscle following nerve crush and transection in order to gain insights into the repair mechanisms following these injuries.

In the current study, transcriptomic and proteomic analysis of muscles were evaluated at 4 weeks after the nerve injury. The different degrees of nerve injury applied to the DN and RN groups appears to result in the different expression profiles of genes and proteins, as well as the muscle repair process. We proved that RN group achieved better functional results concerning the peripheral nerve injury model of rats than DN group. The tibial nerve function of the RN group recovered better with a higher TFI value and better protection for the gastrocnemius muscle. Transection of nerve caused a greater degree of axonal destruction, which required greater recovery and re-innervation at the lesion site. In contrast with transection, nerve crush preserved the nerve sheath...
conduit, so as to allow the axonal regeneration and ultimately evoke a different genes profile. Apart from the Venn-diagrams, volcano plot in Fig. 2c was also demonstrated the different gene profiles between DN and RN groups at 4 weeks post-repair. Furthermore, pathway enrichment analysis based on the KEGG database was performed to evaluate the potential function of DEGs and DEPs. As shown in Fig. 3c, DEPs were significantly enriched in pathways related to energy metabolism, such as fatty acid metabolism, degradation and elongation. Interestingly, the DEPs involved in energy metabolic pathway were mainly distributed in the expression patterns of profiles 5 in STEM analysis (Fig. 3f), which suggest that RN group had a positive effect on energy metabolism comparing to DN group.

When the peripheral nerve is damaged, the distal axons of the injured site degenerates, then Schwann cells of peripheral nerve are able to proliferate and re-differentiate, thus providing an environment for axonal and nerve regeneration [11]. As Zhang et al. reported, Peroxisome proliferator-activated receptors-γ (PPAR-γ) expression in SCs increases during the inflammation process and this may promote their activation as immune cells [12]. In the current study, the PPAR pathway was found to be one of the major signaling pathways according to the multi-omics analyses. They regulate genes that are critical for cellular differentiation, lipid and metabolic homeostasis, and energy balance [13, 14]. PPAR-γ plays a key role in muscle cell function and myogenesis, which expressed in the skeletal muscle of humans [15]. As a nuclear receptor, it can activate cellular metabolism to promote cell growth and differentiation [16, 17]. Given these observations, drugs that activate PPAR-γ signaling pathway may therefore be effective in reducing and preventing muscle atrophy.

Cellular respiration is a series of metabolic reactions that convert biochemical energy to ATP and then release the waste products [18, 19]. Skeletal muscle cells
efficiently converted energy through several metabolic pathways to maintain continuous muscle contraction. In denervated muscle, the process by which mitochondria produce ATP is damaged. Consistent with earlier reports [20], we found lower expression levels for almost all metabolic pathways after nerve denervation. In pathological conditions, the ability to regulate energy metabolism of skeletal muscle is disrupted. This may explain why many pathways associated with energy metabolism were found to be enriched in the current research. Furthermore, the levels of ATP increase rapamycin (mTOR) activity, and the mammalian target of mTOR pathway is the latest intracellular fuel-sensing mechanism to be implicated in the regulation of energy balance [21–23]. In organism, mTOR was a key regulator in maintaining skeletal muscle mass which regulates protein anabolism and catabolism [24]. We have previously proven that negative regulation of mTORC1 signaling could protect the denervated muscle atrophy and improved the functional outcome [25–28]. The essence of denervated muscle atrophy results from more significant muscle protein degradation than synthesis [29, 30]. After denervation, mTORC1 signaling plays more important roles in protein degradation than in protein synthesis and leads to muscle atrophy [24]. Recent studies have also demonstrated that mTORC1 activation increases both oxygen consumption and mitochondrial number [31], it might have a significant role in the coordination of cell growth and energy metabolism. Combined with previous studies, the changes in genes and proteins involved in energy metabolism we investigated in the present study, may be associated with the mTOR pathway, leading to muscle atrophy and thus reduced muscle function.

In the rat model used here, a significant up-regulation of thrombospordin 4 (Thbs4) in RN muscles was observed in the 4-week post-surgery period, which has important functions for neurogenesis in early post-natal and adult rodent brain [32]. Thbs4 could enhance the vesicular trafficking of membrane attachment complexes to stabilize the sarcolemma in myofibers [33]. On the other hand, Thbs4 homozygous knockout mice (Thbs4-KO) resulted in abnormal glial scar formation after injury, and significantly increased microvascular hemorrhage into the brain parenchyma [32]. Loss of Thbs4 expression led to dystrophic changes in the muscular dystrophic mouse models, while overexpression protected muscle and alleviated dystrophic disease.

Other nerve growth factors with increased expression observed in the RN group included Myh3, as well as Postn, Col6a1, and Cfi. Myosin heavy chain 3 (Myh3) gene is an important component of myosin, which is related to the traction and sliding of muscle [34]. It is highly induced during in vitro muscle cell differentiation, and its expression is markedly increased in muscular dystrophy, where it is thought to represent a marker of muscle regeneration [35]. As an important marker gene for muscle regeneration, Myh3 was also found high expression in the RN group in our study, and its expression correlated with the time when most muscle repair occurs [36]. In addition, Myh3 had also been reported to express during fetal development and muscle regeneration [37]. Periostin (Postn) is an extracellular matrix protein secreted by osteoblast and osteoblast-like cell line, which can promote proliferation and differentiation of osteoblast and its precursors on the periosteum [38]. It can not only promote the reconstruction of damaged blood vessels [39], but also promote the hyperplasia and fibrosis of inflammatory tissue [40]. Basing on above evidences, this may be related to the process of muscle repair and nerve regeneration in the RN group.

Collagen type VI alpha 1 chain (Col6a1) is widely distributed in the extracellular matrix of systemic tissues including skeletal muscle. Deficiency of Col6a1 has been shown to cause mitochondrial defects (25–30) and impaired muscle regeneration [41]. As Sakurai et al. showed [42], Col6a1 supplementation promoted muscle regeneration and maturation in Col6a1-KO mice. Thus, the high expression of Col6a1 in RN group might relate to the better muscle recovery in the present study. As for the complement factor I (Cfi), local complement expression by skeletal muscle in vivo may be implicated in some muscular inflammatory or pathological processes. As shown in the above studies, these genes are involved in regulating the muscle development or regeneration in vitro, however, the specific mechanism needs to be further studied and allow them to be the possible test candidates for therapeutic application.

It should be mentioned that there are some limitations in this study. The rats used in the experiments were female at about 8 weeks, which are between the puberty stage and breeding age. The phase of estrous cycle may have an influence on the secretion of various hormones that regulate muscle metabolism. After crushing nerve injury, temporary activity limitations may also affect food intake, which may in turn affect gene and muscle protein expression. However, the rats used in each group are the same age, which can ensure the consistency of each group and minimize the influence of hormonal profile between each group. Another drawback of our study was genes and related pathways were not investigated. The detection of candidate genes and proteins are not enough to support the whole survey for obtaining more comprehensive information. Perhaps, hormone secretion levels of rats and functional analyses of key genes/ proteins could be included in the future study to obtain more comprehensive information.
In spite of the mechanisms of muscle atrophy are still not fully understood, new approaches are now available to comprehensively observe changes in gene and protein expression during skeletal muscle atrophy caused by crush or transection. This has prompted investigations into novel therapeutic and biological solutions to enhance neural repair at an earlier phase. We identified 441 DEGs and 223 DEPs that were specific to the way of nerve injury. Furthermore, several possible candidate genes were identified in the re-innervated nerve, including Thbs4, Myh3, Postn, Col6a1 and Cfi. In particular, the PPAR signaling and energy metabolism pathways seem to play a vital role and warrant further study. This study provides basic information on the changes that occur in gene and protein expression during re-innervation-induced muscle atrophy and identified several key proteins as possible treatment targets. The results form the basis for further research aimed at the prevention and therapy of muscle atrophy.

Methods and materials

Animals
Eighteen adult female Sprague–Dawley rats (eight weeks, 200–250 g) were provided by the Experimental Animal Center of South China University of Technology and randomly divided into 3 groups (n=6) to undergo nerve operations or sham operations. All experiments were carried out in SPF (specific pathogen free) animal house, and the animal feed was formulated based on the nutritional requirements of rats issued by the Ministry of Health.

Surgical procedures
We randomly divided the rats into three groups: the denervated (DN) group, the re-innervated (RN) group, and the control group. Standard aseptic technique was conducting to perform the surgery on the right posterior limb of each animal. Briefly, adult rats were anesthetized with the 30 mg/kg dose of sodium pentobarbital by intraperitoneal injection. All procedures were performed on animals in an unconscious stage on the same day. A longitudinal incision was made posterior to expose the sciatic nerve, and free the tibial and peroneal nerves totally. For relieving post-surgery pain, the rats were given buprenorphine (0.2 mg/kg subcutaneously) when performing the operation.

In the control group, the tibial nerve of rats was exposed clearly without being cut (Fig. 5a). In the DN group, the tibial nerve was transected at 6.5 mm proximal to the gastrocnemius muscle, thus simulating a denervated muscle. In order to prevent spontaneous re-innervation of the tibial nerve, we covered the proximal and distal nerve stumps with a silicone cap (Fig. 5b).
the RN group, the exposed tibial nerve at the same location as the transection site in the DN group was crushed three times for 10 s each using a pair of forceps, with an interval of 10 s. This model simulates a re-innervated muscle (Fig. 5c). The surgical wounds of all rats were thoroughly irrigated and sutured in layers. After the surgical incisions closed, animals were housed in controlled, maintaining a 12-h light/12-h dark cycles in the pathogen-free condition and allowed free access to water and food.

**Analysis of Tibial Function Index (TFI)**

TFI were calculated before and every week after surgery. After dipping the hindlimbs into black ink, the rats walked on the confined walking track (10 cm × 60 cm) covering with white paper. After the test, the footprint on the track was measured with the standard of clarity and completeness. We recorded the paired footprint length (PL, distance from heel to toe), intermediary toe spread (IT, distance from second to fourth toes), and toe spread (TS, distance from first to fifth toes) of each animal’s hind limb. The lengths of normal control left hind leg were defined as NPL, NIT, and NTS, while the experimental right legs were EPL, EIT, and ETS. The TFI analysis was performed following the formula of Bain-Mackinnon-Hunter [43]:

$$\text{TFI} = 104.4 \left( \frac{\text{ETS} - \text{NTS}}{\text{NTS}} \right) - 37.2 \left( \frac{\text{EPL} - \text{NPL}}{\text{NPL}} \right) + 45.6 \left( \frac{\text{EIT} - \text{NIT}}{\text{NIT}} \right) - 8.8$$

**Muscle weight**

Four weeks after the surgery, the animals were sacrificed by the lethal dose of ether. Three operated muscle samples from each group were randomly selected to peel the gastrocnemius muscle and tibial nerve completely. The muscle wet weight was scaled analytical balance (BSA124S, sartorius). The muscle samples were snap-frozen in liquid nitrogen for more than 15 min, and stored at -80°C until bioinformatic analysis 3 days later.

**Transcriptomic analysis**

**RNA extraction, library construction and sequencing**

Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) was used to extract all RNA. Oligo(dT) beads were used to enrich eukaryotic mRNA, while Ribo-ZeroTM Magnetic Kit (Epicentre, Madison, WI, USA) was used to remove rRNA and enrich prokaryotic mRNA. The enriched mRNA was reverse transcribed into cDNA using primers. DNA polymerase I, RNase H, dNTP and buffer were used to synthesize second-strand cDNA, and purified using PCR extraction kit (Qiagen, Venlo, The Netherlands). The sequencing platform of Genedenovo Biotechnology Co., Ltd (Guangzhou, China) is responsible for constructing the library and sequencing it.

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis**

Differential expression analysis of mRNA between two groups was performed using the R package DESeq2, and between two samples by edgeR. Statistically significant differentially expressed genes were detected if parameter of false discovery rate (FDR) set at < 0.05 and an absolute fold change ≥ 2. The KEGG analysis was performed using the KEGG pathway database (Release 94, https://www.genome.jp/kegg/pathway.html) to analyze the selected DEGs at the functional level [44–46].

**Proteomic analysis**

**Protein digestion**

The gastrocnemius muscle samples were put into the lysis buffer (1 mg/ml protease inhibitor, 7 M urea, 2% SDS). The protein concentration of the supernatant was measured using BCA Protein Assay Kit. 50 μg proteins were suspended in 50μL solution, 1uL 1 M dithiotreitol was added for reduction at 55 °C for 1 h, and 5uL 20 mM iodoacetamide was added for alkylation at 37 °C for 1 h. Then 300uL aceton was chilled and precipitated the sample overnight at -20°C. After two washes with cold acetone, the precipitate was resuspended in 50 mM ammonium bicarbonate. Finally, sequence-grade modified trypsin (Promega, Madison, WI) was used to digest the proteins at a substrate/ enzyme ratio of 50:1 (w/w) at 37 °C for 16 h.

**High PH Reverse Phase Separation**

By using high pH separation, the peptide mixture was fractionated on an Ultimate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA), then analyzed on EASY-nLC 1200 system (Thermo Fisher Scientific, MA, USA).

**Data analysis**

Spectronaut X (Biognosys AG, Switzerland) analyzed the raw Data of DIA with default parameters. DEPs with Absolute AVG log2 ratio > 0.58 and q < 0.05 will be filtered after Student’s T-test. KEGG database were annotated to obtain function of proteins, while GO pathways were detected within DEPs with q < 0.05.

**Statistical analysis**

Continuous data were presented as mean ± standard deviation or median with interquartile range.
Two-tailed tests were used, and differences were considered significant if \( p < 0.05 \). For experiments on more than two groups within one categorical variable, ANOVA analysis was performed followed by Tukey’s post hoc test. SPSS 20.0 statistical software (Chicago, IL, USA) was used for the analysis. The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Abbreviations**

- DN: Denervated; RN: Re-innervated; RNA seq: RNA sequencing; SCs: Schwann cells; PPARs: Peroxisome proliferator-activated receptors; DEGs: Differentially expressed genes; DEPs: Differentially expressed proteins; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; Myh3: Myosin Heavy Chain 3; Postn: Periostin gene; Col1α1: Collagen type VI alpha 1 chain; C rit: Complemen-
t factor I; mTOR: Rapamycin.

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**ARRIVE guidelines**

Our study is reported in accordance with ARRIVE guidelines. The ARRIVE Essential 10 are following:

1. **Study design**

   - We divided all animals into 3 groups: denervated group, re-innervated group and control group. Two simulated nerve injury models in the rat tibial nerve were compared: the nerve was either cut (denervated, DN group) or crushed but with the nerve sheath intact (re-innervated, RN group). The control group had a preserved and intact tibial nerve.

2. **Sample size**

   - We provided 6 rats in each group, and a total of 18 rats in 3 groups. We determined the sample size in the previous pre-experiment. The degree of freedom (E) of variance analysis should be estimated, and the value range of E should be between 10-20.

\[ E = \frac{\text{sum of experimental animals in each group} \times 3}{18} - \text{number of groups} \quad (3) = 15. \]

3. **Inclusion and exclusion criteria**

   - All rats underwent rigorous screening by the animal center to ensure their health. We selected the rats randomly to control and treatment groups. Daily observations of animals in each experimental group revealed normal behavior and no systemic or local inflammation or post-operative complications, so all the included rats were analyzed.

4. **Randomization**

   - Randomization was used to allocate rats to control and treatment groups. The rats were randomly divided into 3 groups with 18 rats, which were divided into 3 groups by using random number table. First, the rats were organized into 1, 2, 3... 18, and then arbitrarily from a random number table from a row of a certain number to copy 18 numbers and corresponding. Divide each number by 3 and represent A, B, and C with the remainder 1, 2, and 3.

5. **Blinding**

   - Qingtian Li, Haotao Li, and Yijian Chen designed and conducted the experiment, so they were aware of the group allocation. Bofu Lin, Shuai Wang and Zhantao Deng analyzed and interpreted the data. They were not aware of the group allocation.

6. **Outcome measures**

   - The analysis of Tibial Function Index, muscle weight measurement, and the transcriptome and proteome analysis were described in the “method” part.

7. **Statistical methods**

   - The statistical methods used for each analysis were described in the “method” part.

8. **Experimental animals**

   - Adult female Sprague-Dawley rats (eight weeks, 200–250g) were provided by the Experimental Animal Center of South China University of Technology.

9. **Experimental procedures**

   - The procedure details were described in the “Surgical Procedures” part.

10. **Results**

    - Summary/descriptive statistics for each experimental group were described in the “Result” part.

**Authors’ contributions**

Qingtian Li, Haotao Li, and Yijian Chen designed and conducted the study, and approved the final version of the manuscript. Haotao Li and Qingtian Li wrote the first draft of the manuscript. Weng J, Zhang P, Yin X, and Jiang B modified the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository [47] with the dataset identifier PXD003031.

**Declarations**

**Ethics approval and consent to participate**

Ethics approval and consent to participate All work was performed in accordance with the Guiding Opinions on the Treatment of Experimental Animals, Guangdong Province Laboratory Animal Management Regulations and the Laboratory Animal Committee (LAC) of the South China University of Technology Policy on the Humane Care and Use of Vertebrate Animals. Ethics approval was obtained from the Administration Committee of the Guangdong Provincial People’s Hospital, Guangdong Academy of Medical Sciences (Permit Number: 2018S37A).

**Consent for publication**

The manuscript does not include details, images, or videos relating to an individual person.

**Competing interests**

The study was financially supported by institutional funds only. All authors declare that they have no conflict of interest.

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