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Original Article

Expression of Wnt/β-catenin related genes after skeletal muscle contusion

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Abstract: Background: It was aimed to determine expressions of genes related to Wnt/β-catenin signaling for evaluating time duration after skeletal muscle contusion. Methods: Pathological change of skeletal muscle was observed after H-E staining. mRNA of respective genes was quantified with real-time quantitative PCR. Expression of β-catenin was further characterized with immunostaining and quantified as intensity/area and further immune blotting and quantified as grey intensity normalized to loading control (GADPH). Results: After injury, skeletal muscle exhibited prominent inflammatory response, hyperplasia and regeneration. Infiltration of inflammatory cell, formation of myotube and maturation of skeletal muscle fiber were observed under HE staining. Expression of FZD4, Myo D, Myf5 changed during early stages after injury and could serve to evaluate injury within 24 h; Expression of SFRP5 and Fra1 changed during early-to-intermediate stages after injury and could serve to evaluate injury within 12-48 h; Expression of MRF4 changed during intermediate stages after injury and could serve to evaluate injury within 36-48 h; Expression of β-catenin changed during intermediate stages after injury and could serve to evaluate injury within 36 h-3 d; Expression of MyoG changed during late stages after injury and could serve to evaluate injury within 48 h-7 d. Immunostaining experiments showed that 36 h after injury, membrane β-catenin decreased while nucleus β-catenin increased. Conclusion: Wnt/β-catenin related genes are involved in regeneration of skeletal muscle after contusion. The sequential changes of gene expression can be used for evaluating the duration after contusion.

Keywords: Forensic pathology, injury time estimation, skeletal muscle contusion, Wnt signaling pathway, skeletal muscle satellite cell

Introduction

Time is important in forensic biology and pathology for predicting the time of injury based on which the injury before and after death and the survival duration after injury can be evaluated. This is of great importance for case-reconstruction, clarification of suspects and formation of forensic evidences. Current forensic pathologic examinations for evaluating injury duration include observation with naked eye and microscope, enzymatic chemical examination, biochemical examination, immunohistochemical examination and classic molecular biological assays and diagnostic radiology [1-7].

Current studies on injury time focused on head, skin and spinal cord injuries [3-12], while the situation on skeletal muscle is relatively unknown. Skeletal muscle injury is common in forensic examination especially in cases involving blunt injury and traffic accidents. Regeneration of skeletal muscle is independent of injury type [11], thus could serve as a stable readout of injury time. Skeletal muscles are composed of highly-differentiated and parallel-aligned muscle fibers with multiple nuclei, thus adult skeletal muscle per se cannot regenerate. The key source for regenerating skeletal muscle are a particular cell population on muscle fiber called satellite cells [13]. Regeneration of skeletal muscle is a process involving multiple factors, among which Wnt/β-catenin signaling is a most important component. Wnt/β-catenin is the key factor for terminal differentiation of muscle cells and satellite cells. Activating and inhibiting Wnt signaling can actively modulating satellite cell differentiation. Tras-locating of

Table 1. List of primers and gene ID

Gene	Forward (5'-3')	Reversed (5'-3')	Product	ID
RPL32	CACAGCTGGCCATCAGAGTCA	AAACAGGCACACCAAGCCTCTATTC	83	NM_013226
RPL13	AAGGTGGTGGTTGTACGCTGTG	CGAGACGGGTTGGTGTTCATCC	110	NM_173340
SFRP5	CTCTGGACAACGACCTCTG	CGCATCTTGACCACGAAG	154	NM_001107591.1
FZD4	TGTTCTCAAGTGTGGCTACG	TGGACGAATCAATCAGGAAGG	140	NM_022623.2
β-catenin	TGCGGCTGCTGTTCTATTC	CCATTGGCTCTGTCCTGAAG	104	NM_001330729.1
Myf5	TGAGGGAGCAGGTAGAGAAC	CATCAGAGCAGTTGGAGGTG	84	NM_005593.2
Myo D	CGCTCCAACTGCTCTGATG	TAGTAGGCGGCGTCGTAG	86	NM_176079.1
MRF4	AACTACATTGAGCGTCTGC	AGAATTTCTTGCTTGGGTTTG	107	NM_002469.2
Myo G	CGCTCCAACTGCTCTGATG	TAGTAGGCGGCGTCGTAG	86	NM_176079.1
c-myc	CTTCATTGAGCACATCCTTCG	GTCGGTAGCACACAGAGC	100	NM_001100708
Fra-1	ACAGTCCAGCAGCAGAAG	GGGTCCCAGGAAATGAGG	76	NM_012953.1
Lamin	CTTCGCACGGCTCTCATC	GCTCATCTCCATCCTCTTCC	112	NM_011509534.1

β-catenin can activating expression of a gene set, thus influencing a large population of downstream signaling events.

In this study, we established skeletal muscle contusion model in rat. By probing expression of Wnt/β-catenin related genes at different time points after injury, we aimed to dissect their relationship with muscle repair and injury time. Thus, we may provide theoretical basis for predicting injury time with changes of expression of certain genes.

Materials and methods

Animals

Adult male Wistar rats (n=60, 200±5 g) were from Animal center of Shanxi Medical University and approved by the Ethic Committee. Animals were raised under proper temperature and humidity with 12/12 h light/dark cycles and free-accessed food and water. Animals were grouped into two groups (control and injury) and sacrificed 4 h, 12 h, 24 h, 36 h, 48 h, 3 d, 7 d, 15 d and 21 d after injury (each with 6 animals).

Modeling and muscle sample

Animals were anesthetized with 10% Chloral hydrate (0.35 ml/100 g BW) then fixed uprightly and left hindlimb was stretched outward slightly and de-haired to expose injury site completely. A PVC tube (50 cm) was placed upon and the bottom end of PVC were tightly targeted to calf muscles. A weight (100 g) was placed and blocked in the upper end of PVC and released for free-fall towards calf muscle. Rats were sacrificed with over-dose pentobarbital sodium and skeletal muscles were then dissected from injured sites after different duration (4 h, 12 h, 24 h, 36 h, 48 h, 3 d, 7 d, 15 d and 21 d after injury).

HE staining

After routine automatic dehydration, transparant ation, embedding, slicing (5 μm, Leica), staining and mounting, samples were observed under light-microscope.

RNA extraction, reverse transcription and quantitative PCR

Muscle tissues were frozen under -80°C (Sanyo). Tissues were then lysised with Trizol reagent (Takara). cDNA was made after reverse transcription. 2× TransStart® Tip Green qPCR SuperMix (Transgene) was used for qPCR (CFX 96, Bio-Rad) following manual. Primers for qPCR were designed with AlleleID6.0, tested for specificity with Primer Blast and synthesized by Invitrogen (**Table 1**). Detailed information was listed as followed. Results of qPCR was quantified with $2^{-\Delta\Delta c}$ approach.

Immunohistochemical staining

Muscle tissues were cut into pieces with 0.5 cm diameter, embeded with OCT (Sakura) and cryosectioned (CM1850, Leica) into 3 μm slices and attached onto coverslip for staining. After section, coverslip with slices were fixed in aceton for 15 min, washed with PBS for three times (3 min each). After removing residue liquid with

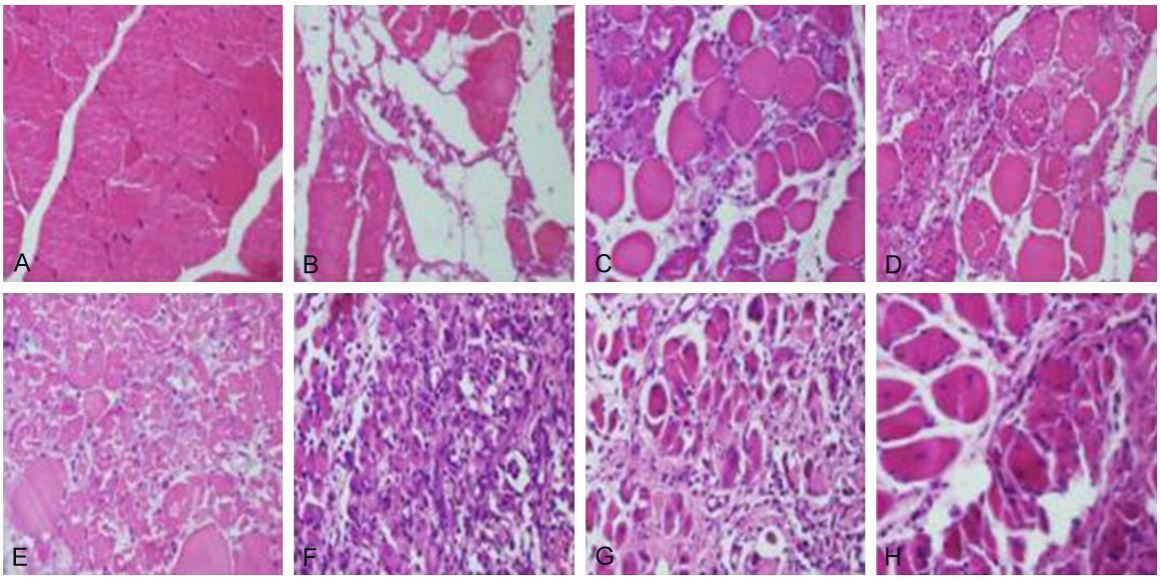

Figure 1. HE staining at different time points after skeletal muscle contusion. A: Control; B: 12 h; C: 24 h; D: 36 h; E: 48 h; F: 7 d; G: 15 d; H: 21 d.

filter paper, 50 μl ketone was added for 10 min at 37°C for permeabilization. Samples were further washed with PBS for 3×3 min and then blocked with 5% fetal donkey serum (Solar Bio) for 30 min at 37°C. Then first antibody against β-catenin (1:100, 50 μl, rabbit anti-rat, Santa) was added and samples were incubated at 4°C overnight (12 h-14 h). Following incubation, samples were allowed to rest in room temperature for 10 min and then in 37°C for 20 min, after washing with PBS (5×5 min), samples were incubated in second antibody (1:200, 50 μl, donkey anti-rabbit, Santa) at 37°C for 30 min. Samples were further washed with PBS (5×5 min) and then incubated with DAPI (1:3, 50 μl, Bioscience) in 37°C for 10 min. After final wash with PBS (3×3 min) and removing residue liquid, samples were covered with anti-fade medium (Beyotime Bio) and coverslip for observation under fluorescence microscope (BX61, Olympus).

Image processing and quantification

Images were analyzed with Image 6.0 software for calculating the ratio of fluorescent intensity/area. Results were exported automatically.

Immunoblotting

Total protein was extracted with ProteoPrep® (Sigma) following standard protocol. Protein concentration was quantified with BCA method (Sigma) based on which concentration of total protein was adjusted to 4 mg/ml. Samples were then mixed with 5× loading buffer and incubated in boiled water for 10 min. Part of samples were stored in 4°C for immediate electrophoresis and remaining samples were stored in 4°C for further use. Samples were loaded for electrophoresis (5% concentrating and 12% separating gel) and then transferred to nitrocellulose membrane (NC membrane). Further steps including blocking, antibody incubation, washing and visualization followed standard procedures. Protein was quantified as integrate optical density (IOD) normalized to the value of GADPH.

Statistics

SPSS 16.0 was used for data analysis. Quantitative results were represented as mean ± sd. Differences between groups are tested with student *t* test. Significance was accepted if $P < 0.05$.

Results

Evaluating modeling with HE staining

In normal control animal, muscle fiber exhibit regular and tight aligned multi-edge shape. Striation are evident with pink cytoplasm and oval nuclei underneath intact muscle membrane without bleeding, necrosis or infiltration

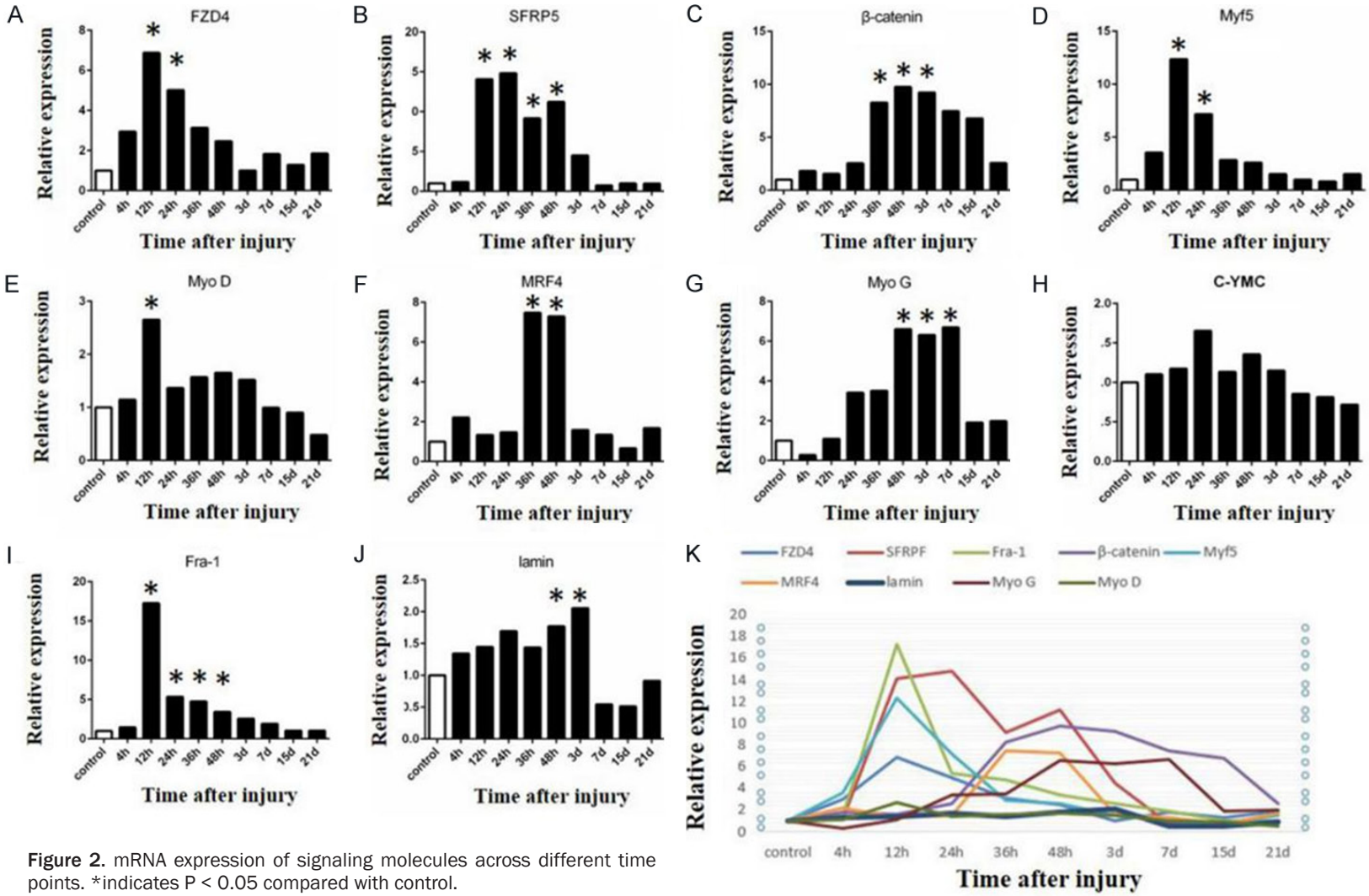

Figure 2. mRNA expression of signaling molecules across different time points. *indicates $P < 0.05$ compared with control.

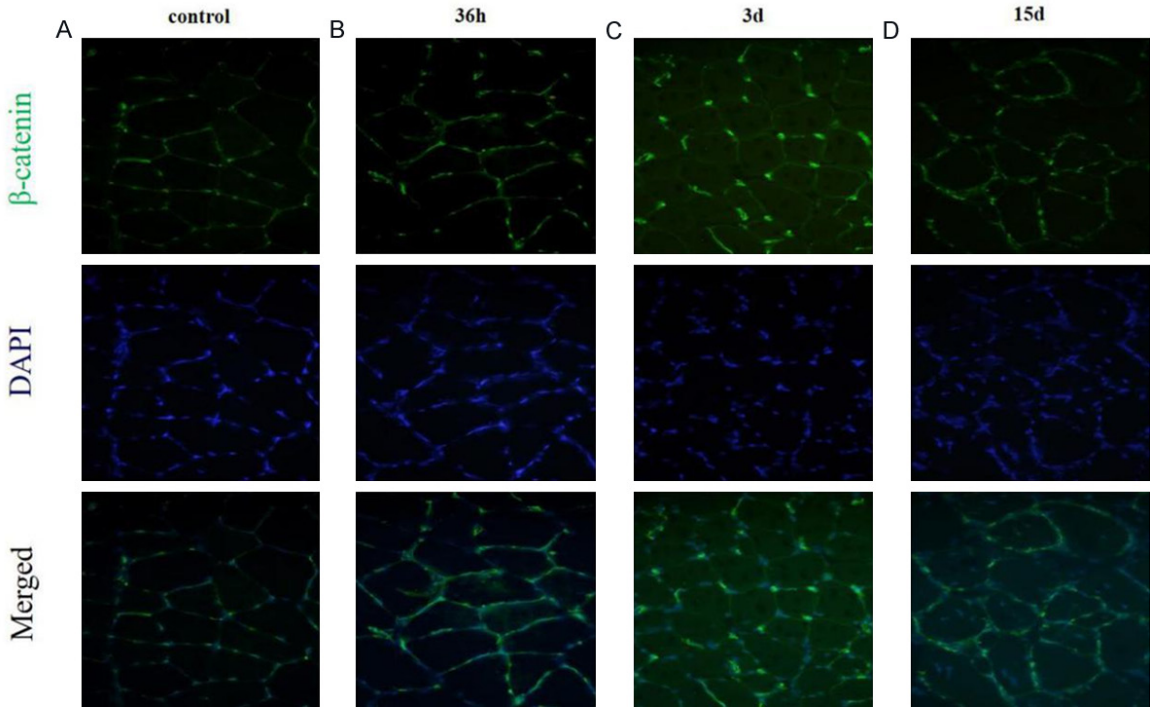

Figure 3. Distribution of β-catenin across different time points.

of inflammatory cells (**Figure 1A**). 12 h after contusion, different levels of necrosis were evident mesenchymal infiltration of red cell and neutrophil are observed with proliferation of fibrous tissue (**Figure 1B**). Mesenchymal infiltration of inflammatory cells (mainly neuropil and clasmatoblast) was more evident 24 h (**Figure 1C**) and progressed more severely until 36 hours after contusion (**Figure 1D**). 48 h after contusion, neuropil infiltration was reduced, but, macrophage infiltration became obvious and necrosis of some muscle fiber, disappearance of nuclei and proliferation of connective tissues are evident (**Figure 1E**). 7 d after contusion, around injury site, necrosis of most muscle fiber, proliferation of connective tissues, accumulation of nuclei-structures (most from skeletal muscle nuclei and part from fibroblasts) are evident with some macrophage, lymphocytes and muscle fiber with central nuclei and appearance of new myotube (**Figure 1F**). 15 d after contusion, central nuclei formation became more evident and the number of central muscle fiber increased obviously and fused to multi-nuclei myotube; The number of inflammatory cells decreased obviously (**Figure 1G**). 21 d after contusion, central nuclei formation was more evident than 15-day, membrane translocation of nuclei were evident, indicating repairmen of injured skeletal muscle (**Figure 1H**).

mRNA expression of signaling molecules

Expression of FZD4 (frizzled class receptor 4) increased and peaked at 12 h then decreased to normal value at 3 d (**Figure 2A**). Expression of SFRP5 increased, peaked at 24 h and decreased to normal value at 7 d (**Figure 2B**). Expression of β-catenin increased and peaked at 48 h and remained higher than control across different time points (**Figure 2C**). Expression of Myf5 (myocyte differentiation factor-5) increased and peaked at 12 h then decreased to normal value at 7 d (**Figure 2D**). Expression of MyoD (myogenic determination factor) increased and peaked at 12 h then decreased to normal value at 15 d (**Figure 2E**). Expression of MRF4 (muscle regulatory factor 4) remain same within 24 h but increased and peaked at 36 to 48 h then decreased to normal value at 3 d to 21 d (**Figure 2F**). Expression of Myo G (myogenin) decreased at 4 h then increased to and remained peak from 48 h to 7 d; The expression then decreased but still higher than control at 21 d (**Figure 2G**). Expression of c-myc increased and peaked at 24 h then decreased to the value lower than control at 7 d (**Figure**

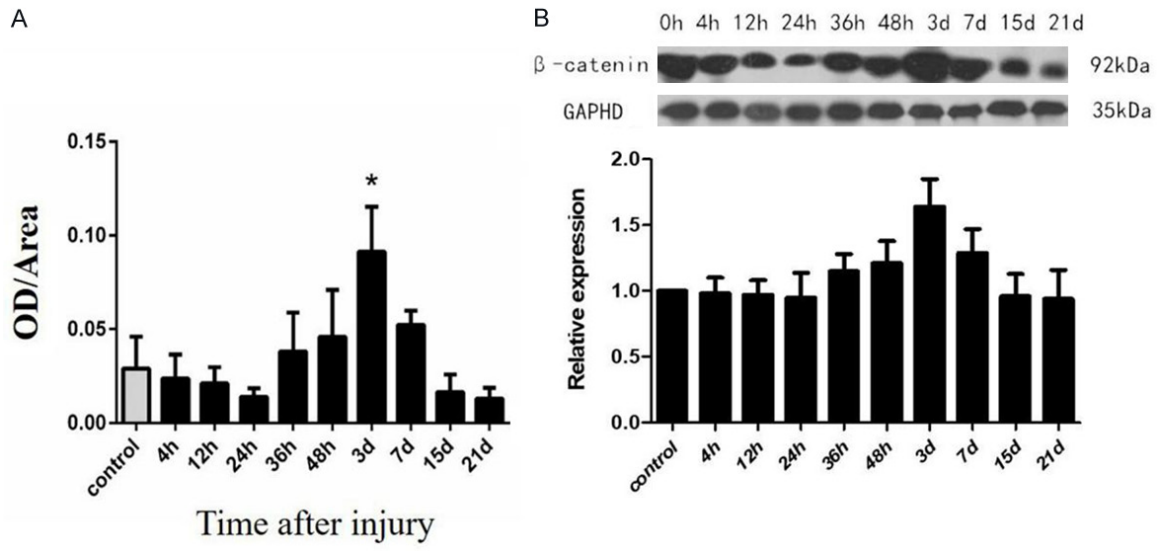

Figure 4. Expression of β-catenin protein quantified from immunostaining and immunoblotting across different time points. *indicates P < 0.5 compared with control.

2H). Expression of Fra1 (Fos-related antigen 1) increased and peaked at 12 h and decreased gradually to normal value at 15 d (**Figure 2I**). Expression of Lamin increased and peaked at 3 d and remained significantly higher at 48 h and 3 d; The expression decreased gradually to the level even lower than control and recovered to control level at 21 d (**Figure 2J**). In summary, expression-changes of FZD4, MyoD and Myf5 are evident in early stage (within 24 h), expression changes of SFRP5 and Fra1 are evident in early-intermediate stage (12 h to 48 h), expression change of MRF4 is evident in intermediate stage (36 h to 48 h), expression change of β-catenin is evident at intermediate stages (36 h to 3 d), and expression change of MyoG is evident in late stage (48 h-7 d). **Figure 2K.**

Distribution of β-catenin

In control sample, β-catenin distributed on the membrane of skeletal muscle (**Figure 3A**); 36 h after contusion, β-catenin was observed both on membrane and in nuclei (**Figure 3B**); 3 d after contusion, membrane-β-catenin decreased and nuclei-β-catenin increased (**Figure 3C**) and 15 d after contusion, β-catenin distributed both on membrane and nuclei, but the nucleus distribution is barely seen (**Figure 3D**).

Protein expression of β-catenin

By quantify fluorescent signals of β-catenin, we found that the expression of β-catenin protein exhibit decrease-increase-decreased mode and peak at 3 d (**Figure 4A**). This result is consistent with immunoblotting experiment (**Figure 4B**).

Discussion

We successfully established rat model for skeletal muscle contusion and tested the expression of molecules related to Wnt/β-catenin signaling. Our finding revealed that expression-changes of FZD4, MyoD and Myf5 are evident in early stage (within 24 h), expression changes of SFRP5 and Fra1 are evident in early-intermediate stage (12 h to 48 h), expression change of MRF4 is evident in intermediate stage (36 h to 48 h), expression change of β-catenin is evident at intermediate stages (36 h to 3 d), and expression change of MyoG is evident in late stage (48 h-7 d). Thus, expression changes of different molecules may be applied to predict the time-duration after injury. Immunostaining and immunoblotting further reveal the dynamic spatial and amount changes of β-catenin, further indicate its active involvement in injury and regeneration.

Repairment of skeletal muscle can be divided into acute, repairment and remodeling phases [14-16] that involving complex cellular and chemical interactions. The whole process includes necrosis of injured muscle fiber, accumulation of inflammatory cells, differentiation of myoblast, fusion for new muscle fiber and fabrication of fibroblast progenitor [17]. Infiltr-

ation of macrophage and neutropil dominant the first phase and inflammatory factor play important roles [13, 18]. In the second phase, growth factors that can stimulating the proliferation and differentiation of satellite cells play important roles and essential for regeneration of injured skeletal muscle [19-21]. Such dynamic processes were also reproduced in our model; thus our model is suitable to simulate the biological processes underlying repairment of injured skeletal muscle.

Wnt signaling is important for maintaining homeostasis, proliferation and differentiation of stem cell [22]. Previous study showed that activation of Wnt is essential for full regeneration of skeletal muscle [23]. In adult rat, Wnt activation is correlated with transformation of satellite cells from muscle-derived cell lines [24]. Wnt is also essential for muscle formation during embryonic development [25] and is key factor for satellite-fate determination during terminal differentiation of myoblast. In the meantime, Wnt can also induce muscle regeneration from CD45+ stem cell. Thus, activation of Wnt signaling is a possible cause for regeneration and repairment of injured muscle. Such a notion is also supported by our results showing that both the amount and cellular location of Wnt signaling is dynamically controlled during repairment of skeletal muscle (**Figures 3** and **4**).

Increased secretion of Wnt after injury can lead to activation of FZD4, thus in early stage, FZD4 increase, but continuous increased Wnt may bind FZD44 and lead to decrement of FZD4; As an endogenous inhibitor of Wnt signaling [26], SFRP5 may increase in early stage in response to Wnt increment to maintain biological homeostasis while in late phase SFRP5 may decrease as result of Wnt decrement in late stages; β-catenin is the classical factor in Wnt signaling, in response to Wnt increase and the inhibitory effect of SFRP5 (12 h), increment of β-catenin may be delayed temporally (24 h); Expression of Myf5 and Myo D both peaked at 12 h and decreased to normal value at 7 d, indicating that they are expressed in and can activate inactive muscle satellite cells to stimulate muscle differentiation and myoblast fusion [26-28]. Increase of MRF4 in intermediate phase and MyoG in intermediate-late phases indicate that injury can stimulate muscle satellite cell to express Myo D and/or Myf5 and MyD to induce expression of MRF4 and MyoG in skeletal muscle and promote myotube maturation for fiber maturation in late stages. Expression of Fra1 is low in control as in normal condition, skeletal muscle is uncappable of growth, differentiation, proliferation of apoptosis; as a downstream target of Wnt, Fra-1 increased 24 h, 36 h and 48 h after injury, suggesting such increment is related to Wnt activation. Expression of c-myc peaked at early stage after injury, indicating the elevation may not induced through Wnt. Expression of Lamin increased and peaked at 3 d, indicating that they are induced via Wnt signaling and play some role during repairment of skeletal muscle.

In summary, we established skeletal muscle contusion model in rat and characterized dynamic changes of expression of key molecule involved in repairment of skeletal muscle. Our finding revealed that changes of expression of different molecules can indicate particular phase after injury. Thus, by monitoring expression of molecules with routine molecular biological assays, the time duration after skeletal muscle injury can be predicted.

Disclosure of conflict of interest

None.

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