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Fatty acid-binding protein 4 regulates fatty infiltration after rotator cuff tear by hypoxia-inducible factor 1 in mice

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Abstract

Background Fatty infiltration in skeletal muscle is directly linked to loss of muscle strength and is associated with various adverse physical outcomes such as muscle atrophy, inflammation, insulin resistance, mobility impairments, and even mortality in the elderly. Aging, mechanical unloading, muscle injury, and hormonal imbalance are main causes of muscle fat accumulation, and the fat cells are derived from muscle stem cells via adipogenic differentiation. However, the pathogenesis and molecular mechanisms of fatty infiltration in muscles are still not fully defined. Fatty acid-binding protein 4 (FABP4) is a carrier protein for fatty acids and is involved in fatty acid uptake, transport, and lipid metabolism. Rotator cuff tear (RCT) usually occurs in the elderly and is closely related with fatty infiltration in injured muscle. To investigate potential mechanisms for fatty infiltration other than adipogenic differentiation of muscle stem cells, we examined the role of FABP4 in muscle fatty infiltration in an RCT mouse model.

Methods In the RCT model, we evaluated the expression of FABP4 by qRT-PCR, western blotting, and immunohistochemical analyses. Histological changes such as inflammation and fat accumulation in the injured muscles were examined immunohistochemically. To evaluate whether hypoxia induces FABP4 expression, the levels of FABP4 mRNA and protein in C3H10T1/2 cells after hypoxia were examined. Using a transient transfection assay in 293T cells, we assessed the promoter activity of FABP4 by hypoxia-inducible factors (HIFs). Additionally, we evaluated the reduction in FABP4 expression and fat accumulation using specific inhibitors for HIF1 and FABP4, respectively.

Results FABP4 expression was significantly increased after RCT in mice, and its expression was localized in the intramuscular fatty region. Rotator cuff tear-induced FABP4 expression was up-regulated by hypoxia. HIF1α, which is activated by hypoxia, augmented the promoter activity of FABP4, together with HIF1β. Hypoxia-induced FABP4 expression was significantly decreased by HIF1 inhibitor treatment. Furthermore, in RCT model mice, fat accumulation was remarkably reduced by FABP4 inhibitor treatment.

Conclusions This study shows that RCT induces FABP4 expression, leading to fat accumulation in injured muscle. FABP4 transcription is regulated by the direct binding of HIF1 to the FABP4 promoter in the hypoxic condition induced by RCT. Fat accumulation in injured muscle was reduced by the inhibition of FABP4. Ultimately, in the RCT model, we identified a novel mechanism for fatty infiltration by FABP4, which differs from adipogenic differentiation of muscle stem cells, and we found that fatty infiltration might be regulated by inhibition of HIF1 or FABP4.

Keywords Muscle fatty infiltration; Rotator cuff tear; Fatty acid binding protein 4; Hypoxia-inducible factors

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Introduction

Among various musculoskeletal complaints, rotator cuff tear (RCT) is one of the representative muscle injuries leading to fatty infiltration and muscle loss in aged individuals.[1–5] Muscle atrophy and fatty infiltration are major deleterious phenomena in RCT and are closely correlated with poor functional outcome following even adequate repair because they have been considered irreversible phenomena.[1,6,7] Therefore, RCT could be considered a good model to understand age-associated muscle fatty infiltration. To date, the mechanism of muscle fatty infiltration in RCT has been investigated using various animal models.[8–13] However, the molecular mechanisms underlying rotator cuff muscle atrophy and fatty infiltration remain largely unknown.

Muscle fatty infiltration is closely associated with muscle atrophy, inflammation, insulin resistance, mobility impairments, fracture, and even mortality in the elderly.[14–17] Although the factors leading to fat accumulation in the muscle are not yet fully defined, it has been reported that aging, muscle injury, mechanical unloading, and hormonal imbalance are main causes of ectopic fat formation.[1,2,18–20] With respect to the molecular mechanism underlying muscle fat accumulation, it has been reported that pluripotent stem cells or progenitor cells residing in the muscle differentiate into adipocytes by the action of adipogenic transcription factors such as CCAAT/enhancer binding protein alpha (C/EBPα) and peroxisome proliferator activated receptor gamma (PPARγ).[8,21,22] Previous studies have suggested that rotator cuff fatty infiltration is also mediated by these adipogenic transcription factors.[8,23–25] However, considering the population of stem cells available at old age, it is possible that other regulators are also involved in intramuscular fat accumulation.

Fatty acid-binding protein 4 (FABP4), also known as aP2, is a member of the intracellular FABP family that bind long-chain fatty acids and other hydrophobic ligands[26,27] and is involved in fatty acid uptake, transport, and metabolism.[28,29] Interestingly, in the livestock field, it is known as a key candidate gene for meat marbling, which is a phenomenon quite similar to muscle fatty infiltration.[30,31] Genetic ablation and pharmacological inhibition studies have shown that FABP4 plays a critical role in many aspects of the development of metabolic diseases and have suggested FABP4 as a therapeutic target for such diseases.[29,32–34] Although FABP4 is assumed to be adipose-tissue specific and has for this reason been used as an adipocyte marker, several studies have reported FABP4 protein expression in skeletal muscle,[35,36] suggesting an additional biological role of FABP4 in the muscle.

Here, we hypothesized that, as a fat-binding protein, FABP4 may play a role in regulating ectopic fat accumulation after rotator cuff injury. To examine this hypothesis, we investigated the expression of FABP4, searched for the upstream regulator, and evaluated the effect of inhibitors of the involved molecules on fat accumulation in a mouse RCT model.

Materials and methods

Establishment of the rotator cuff tear mouse model

Male 8-week-old C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) and kept in a specific pathogen-free facility. Prior to experiments, the mice were acclimatized to a 12 h light/dark cycle at 22 ± 2°C for 1 week and allowed unlimited access to food and water. The mice were randomly divided into three treatment groups (1, 2, and 4 weeks after RCT; n = 10/group) and received unilateral complete supraspinatus tendon transection under anesthesia using Zoletile (Virbac, Carros, France) and Rompun (Bayer Korea Ltd., Seoul, Korea). In the left shoulder, a skin incision was made to expose the rotator cuff tendons. The supraspinatus tendon was exposed and cut from the greater tuberosity of the humerus. The supraspinatus muscle was partly detached from the scapular fossa. A 3–0 nylon suture was used to close the skin, and the mice were allowed unrestricted cage activity. At 1, 2, and 4 weeks after surgery, mice were sacrificed by cervical dislocation, and the supraspinatus muscles were completely separated from the scapular fossa. The muscle samples were used for total RNA or protein extraction (n = 7), and for immunohistological analyses (n = 3) (*Figure* 1). The contralateral supraspinatus muscle was used as a control. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Konkuk University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

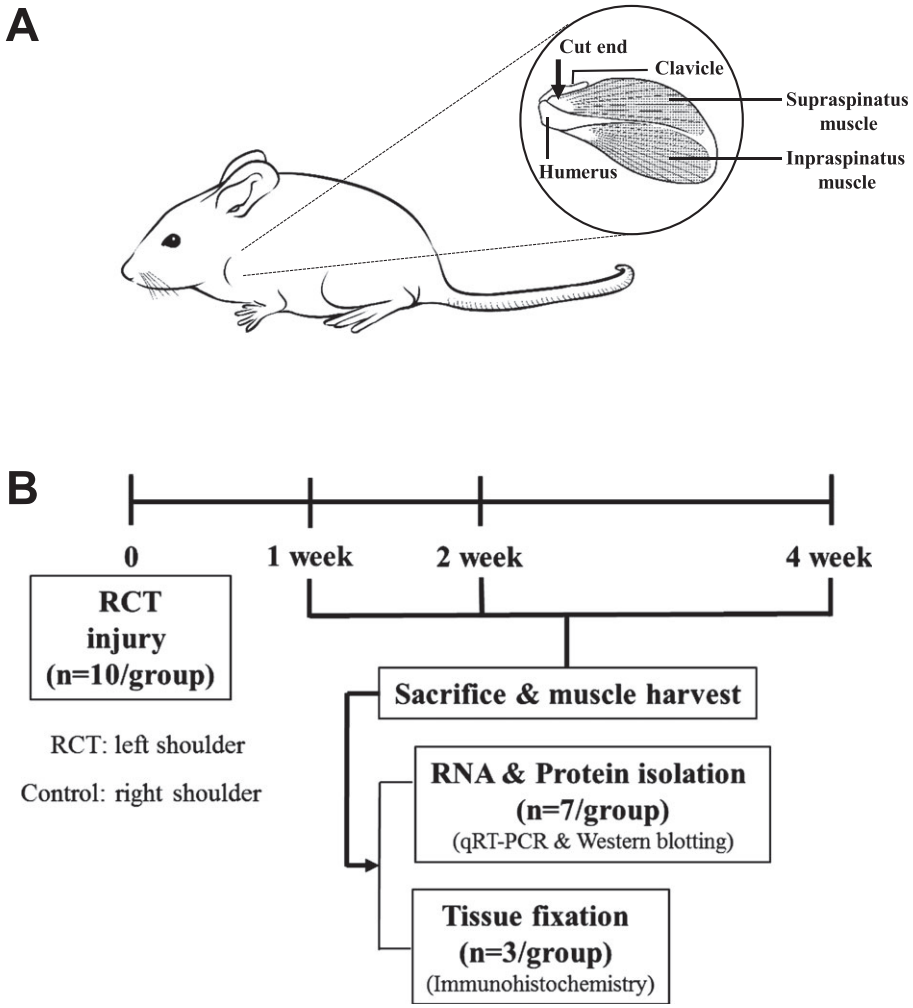
Quantitative reverse transcription (qRT-)PCR analysis

Total RNA was extracted from the isolated supraspinatus muscles or C3H10T1/2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and cDNA was generated using Maxime RT PreMix Kit (iNtRON Biotechnology, Korea). qPCR was carried out on the Light Cycler 480 System (Roche Diagnostics, Swiss) with 2× qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK). All data were normalized to actin expression. The sequences of the primers are listed in *Table* 1.

Western blot analysis

Whole cell extracts from isolated muscle tissue or cultured cells were prepared using radioimmunoprecipitation assay buffer (Elpis-Biotech, Korea). Proteins from the whole cell

Figure 1 Scheme showing the rotator cuff tear (RCT) injury and experimental procedures in mice. *(A)* Complete supraspinatus tendon transection. In the left shoulder, a skin incision was made to expose the rotator cuff tendons. The supraspinatus tendon was exposed and cut from the greater tuberosity of the humerus. The supraspinatus muscle was partly (± 5 mm) detached from the scapular fossa. A 3–0 nylon suture was used to close the skin, and the mice were allowed unrestricted cage activity. *(B)* Overall experimental schedule. Thirty mice were randomly divided into three experimental groups (1, 2, and 4 weeks after rotator cuff tear, n = 10/group) and received unilateral complete supraspinatus tendon transection under anesthesia. At 1, 2, and 4 weeks after surgery, mice were sacrificed by cervical dislocation, and the supraspinatus muscles were completely removed from the scapular fossa. The muscle samples were used for total RNA and protein extraction (n = 7) and for immunohistological analyses (n = 3), respectively. The contralateral supraspinatus muscle was used as a control.



lysates were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were probed with an anti-FABP4 or an anti-β actin antibody (Novus Biochemicals, USA). Immunoreactive proteins were visualized using an Amersham ECL kit (GE Healthcare, NJ, USA), according to the manufacturer's instructions. The protein amounts were assessed by densitometry using Image J software.

Immunohistochemistry

The supraspinatus muscles were dissected in axial cut, 5 mm medial from the musculotendinous junction at the designated time point after surgery, and fixed in neutral buffered 10% formalin (pH 7.4). For immunohistochemistry experiments, 5 μm paraffin-embedded tissue sections were made and deparaffinized in xylene and rehydrated in an ethanol/water series. Antigen retrieval was conducted using citrate buffer (pH 6.0). The slides were incubated with anti-FABP4 (Novus Biochemicals) for 1 h at room temperature, washed 3 times with phosphate-buffered saline, and incubated with the corresponding secondary antibody conjugated to horseradish peroxidase for 30 min at room temperature. The other tissue slides were subjected to haematoxylin and eosin staining. All slides were analysed under an Eclipse Ni-U microscope (Nikon, Tokyo, Japan), and images were

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Table 1 Primers used for quantitative RT-PCR

Gene full name	Gene symbol (mouse)	Sequences; Forward (F)/Reverse (R)
Fatty acid binding protein 4	Fabp4	(F) 5′-AAGGTGAAGAGCATCATAACCCT-3′ (R) 5′-TCACGCCTTTCATAACACATTCC-3′
Fatty acid binding protein 3	Fabp3	(F) 5′-ACCTGGAAGCTAGTGGACAG-3′ (R) 5′-TGATGGTAGTAGGCTTGGTCAT-3′
Interleukin 1 beta	Il-1b	(F) 5′-GAATCTATACCTGTCCTGTG-3′ (R) 5′-ACGGATTCCATGGTGAAGTC-3′
Interleukin 6	Il-6	(F) 5′-AACGATGATGCACTTGCAGA-3′ (R) 5′-GAGCATTGGAAATTGGGGTA-3′
CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa	(F) 5′-AAGAAGTCGGTGGACAAGAAC-3′ (R) 5′-GTCATTGTCACTGGTCAGCTC-3′
Peroxisome proliferator activated receptor gamma	Pparg	(F) 5′-CGGTTTCAGAAATGCCTTGC-3′ (R) 5′-ATCTCCGCCAACAGCTTCTC-3′
Myogenic differentiation 1	Myod1	(F) 5′-CAAGCGCAAGACCACCAACG-3′ (R) 5′-ATATAGCGGATGGCGTTGC-3′
Myogenic factor 5	Myf5	(F) 5′-CCTCATGTGGGCCTGCAAAG-3′ (R) 5′-CATTCCTGAGGATCTCCACC-3′
Vascular endothelial growth factor	Vegf	(F) 5′-GCACATAGAGAGAATGAGCTTCC-3′ (R) 5′-CTCCGCTCTGAACAAGGCT-3′
Actin, beta	Actb	(F) 5′-TCTGGCACCACACCTTCTAC-3′ (R) 5′-TCGTAGATGGGCACAGTGTGG-3′

acquired with a Nikon DS-Ri1 and analysed using NIS Elements F4.00.00 version 4.0.

DNA constructs and transient transfection assay

Reporter plasmids harbouring the mouse *Fabp4* promoter, *Fabp4*-Luc (−5.4 ~ −1.0) and *Fabp4*-Luc (−5.4 ~ −4.9) were kindly provided by Prof. J. Ko (Division of Life Sciences, Korea University, Korea). The DNA constructs used for overexpression of hypoxia-inducible factor 1 (HIF1)α and HIF1β, pcDNA3-HIF-1α, and pcDNA3-ARNT were kindly provided by Dr. Eric Huang (University of Utah School of Medicine). Transient transfections were carried out in 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were co-transfected with a reporter plasmid (0.2 μg/well) and the indicated expression vector. A pCMV-β-galactosidase plasmid (0.1 μg) was co-transfected as an internal control. The total amount of DNA was adjusted to 0.7 μg/well by addition of empty vector, pcDNA3. Cells were harvested 24 h after transfection. Luciferase activity was normalized to β-galactosidase activity. Data are representative of at least three independent experiments.

Reagents and hypoxic challenge

C3H10T1/2 (mouse fibroblast) cells were treated with HIF1 inhibitor (InSolution™ HIF-1 inhibitor, Cat# 400092; Calbiochem, USA) at 10 or 20 μM. The cells were incubated in a hypoxic chamber for 48 h in the presence or absence of HIF1 inhibitor. Dimethyl sulfoxide (DMSO) was used as a vehicle. FABP4 inhibitor (BMS309403, Cat#10010206; Cayman, USA) was dissolved in the manufacturer-recommended solvent, DMSO. One week after RCT, mice were treated daily with BMS309403 by oral gavage (15 mg/kg) for 2 weeks. Dimethyl sulfoxide was used as a vehicle. For hypoxic challenge, C3H10T1/2 cells were incubated in a hypoxic chamber (0.1% O_2, 5% CO_2, 10% H_2, 85% N_2) for the designated period.

Cell culture

C3H10T1/2 cells were cultured in Roswell Park Memorial Institute 1640 medium (BioWest, Nuaille, France) supplemented with 10% foetal bovine serum and antibiotics (BioWest). 293T (human embryonic kidney cells) cells were maintained in Dulbecco's modified Eagle's medium (Corning, USA) supplemented with 10% foetal bovine serum and antibiotics. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Statistical analysis

Data are expressed as means ± standard errors. Means were compared using the Mann–Whitney *U* test. Differences were considered significant at $P < 0.05$.

Results

Fabp4 gene expression is induced by rotator cuff tear in mice

To assess whether FABP4 is regulated by muscle injury, we established an RCT model in mice by injuring the

supraspinatus tendon by complete detachment (*Figure* 1a) and examined changes in the injured muscle at the designated time points (*Figure* 1b). As shown in *Figure* 2a, *Fabp4* gene expression was significantly up-regulated from 2 weeks and sustained for 4 weeks after RCT. Interestingly, the mRNA level of *Fabp3*, the muscle type of FABP,[37] showed no noticeable change (*Figure* 2b). In general, RCT causes profound changes in muscles such as fatty infiltration, muscle degeneration, and atrophy.[38] To validate our mouse RCT model, we analysed the expression of several genes associated with inflammation, adipogenesis, and myogenesis. mRNA expression of the inflammation-associated genes *Il-1b* and *Il-6* was remarkably up-regulated from 1 week after RCT (*Figure* 2c, d), indicating an acute inflammatory response at the early stage following injury. The adipogenic genes *Cebpa* and *Pparg* were up-regulated from 2 weeks after RCT onwards (*Figure* 2e, f). The expression of myogenic regulators *Myod1* and *Myf5* was also significantly increased at 2 weeks after RCT as compared with the control; however, the expression of both genes was reduced 4 weeks later (*Figure* 2g, h). Taken together, these results suggested that muscle injury induces expression of *Fabp4* as well as other muscle injury-related regulators.

Muscle injury-induced fatty acid-binding protein 4 protein expression is localized in the intramuscular fat depot

To confirm protein expression of FABP4, we performed western blot analysis using an anti-FABP4 antibody. As shown in *Figure* 3a, FABP4 protein expression was not altered at the early stage of RCT. However, expression strongly increased with time after the surgery, suggesting that the expression of FABP4 is regulated by RCT. These results corroborated the above qRT-PCR data. Immunohistochemical analysis showed a remarkable inflammatory response in the initial stage (1 week) after RCT (*Figure* 3b). In this stage, there was no noticeable fat accumulation in the injured region, and FABP4 expression was not observed clearly. At 2 weeks after RCT, inflammation was still observed, while partial fat accumulation was revealed. Interestingly, FABP4 expression was localized in the newly formed fatty region (*Figure* 3c). At 4 weeks after RCT, there was obvious fat accumulation in the injured muscle, together with strong FABP4 expression (*Figure* 3d). These results indicated that the expression of FABP4 is up-regulated by RCT and might be associated with fat accumulation.

Fabp4 gene expression is regulated by hypoxia-inducible factor 1 in hypoxia

Regarding the molecular mechanism underlying FABP4 induction by muscle injury, we considered the previous studies reporting that RCT leads to hypoxic condition.[39,40] Therefore, we hypothesized that FABP4 was regulated by hypoxia. To verify this idea, we examined the expression of FABP4 in C3H10T1/2 mouse fibroblasts, which were cultured in a low-oxygen condition. As shown in *Figure* 4a, expression of *Fabp4* as well as the positive control *Vegf* (*Figure* 4b) was significantly induced in a time-dependent manner. Accordingly, western blot analysis revealed an increase in FABP4 protein expression by hypoxia (*Figure* 4c). Hypoxia-inducible factor 1 is activated by hypoxia and regulates various hypoxia-inducible genes together with HIF1b (also termed ARNT). To investigate whether HIF1 activates *Fabp4* transcription, we performed a transient transfection assay in 293T cells using *Fabp4* promoter-driven luciferase reporter constructs. For the *Fabp4*-Luc (-5.4 ~ -1.0) reporter construct harbouring three HIF1-binding sites, HIF1α alone or HIF1α and HIF1β complex significantly augmented reporter activity (*Figure* 4d). However, for the reporter construct *Fabp4*-Luc (-5.4 ~ -4.9) lacking HIF1-binding sites, HIF1 complex did not activate promoter activity (*Figure* 4e). Taken together, these results indicated that muscle injury-induced hypoxic condition regulates FABP4 expression through direct binding of HIF1 to the *Fabp4* promoter.

Hypoxia-induced fatty acid-binding protein 4 expression and muscle injury-mediated fat accumulation are reduced by inhibition of hypoxia-inducible factor 1 and fatty acid-binding protein 4

Finally, we used inhibition assays to confirm that fat accumulation after rotator cuff injury is regulated by HIF1 and FABP4. We examined the effect of HIF1 suppression on FABP4 expression in C3H10T1/2 cells after hypoxic challenge. As shown in *Figure* 5a, the hypoxia-induced *Fabp4* gene expression was significantly decreased by HIF1 inhibitor treatment. Similarly, western blot data showed a remarkable decrease in FABP4 protein expression upon HIF1 inhibitor treatment (*Figure* 5b). Furthermore, in the RCT mouse model (*Figure* 5c), immunohistochemical analysis revealed that the ectopic fat accumulation by RCT was drastically reduced by FABP4 inhibitor (*Figure* 5d). Taken together, these results suggested that, after RCT, hypoxia-mediated fat accumulation can be regulated by inhibition of HIF1 or FABP4 *in vivo*.

Discussion

In the present study, we identified FABP4 as a novel regulator of intramuscular fat accumulation and elucidated the molecular mechanism for its regulation by RCT in mice. As

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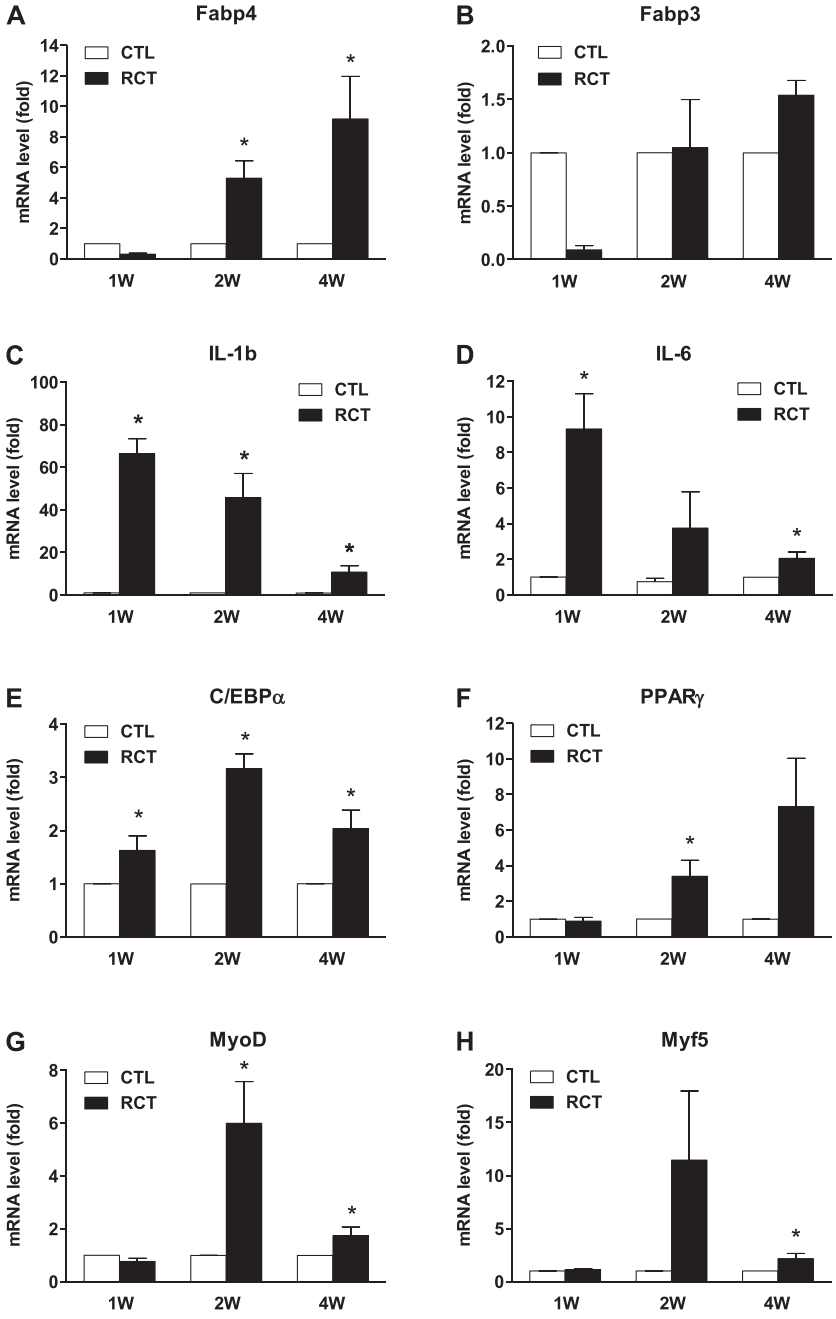
Figure 2 Gene expression profile after rotator cuff tear in mice. At 1, 2, and 4 weeks after rotator cuff tear surgery, mice (n = 7) were sacrificed by cervical dislocation, and the supraspinatus muscles were completely removed from the scapular fossa. The muscle samples were used for total RNA isolation. Quantitative reverse transcription-PCR was performed with mouse primers for *Fabp4 (A)*, *Fabp3 (B)*, *Il-1b (C)*, *Il-6 (D)*, *Cebpa (E)*, *Pparg (F)*, *Myod1 (G)*, and *Myf5 (H)* (refer to *Table* 1). The contralateral supraspinatus muscle was used as a control. CTL: control; RCT: rotator cuff tear. Differences were considered significant at $P < 0.05$.



hypothesized, RCT caused up-regulation of *Fabp4* mRNA and protein expression. Interestingly, FABP4 protein expression was localized in the newly formed fatty region in the injured muscle. At the gene regulation level, *Fabp4* gene expression was regulated by HIF1, a hypoxia-dependent transcription factor. We also observed that the hypoxia-induced FABP4 expression was significantly decreased by HIF1 inhibitor. Furthermore, in an *in vivo* model, fat accumulation in injured muscle was remarkably reduced by FABP4 inhibitor, suggesting that the application of an inhibitor or suppressor of HIF1 or FABP4 would be one of potential therapeutic approaches.

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Figure 3 Muscle injury-induced fatty acid-binding protein 4 protein is expressed in the intramuscular fat depot. At 1, 2, and 4 weeks after rotator cuff tear surgery, mice were sacrificed, and the supraspinatus muscles were isolated for immunohistochemistry. *(A)* Fatty acid-binding protein 4 protein expression in isolated muscles at different stages after rotator cuff tear (n = 4). Densitometric analyses of the western blots are shown in the graphs on the right. Data are mean ± SEM. *$P < 0.05$. *(B–D)* Immunohistochemical analyses of the isolated muscle at 1 week *(B)*, 2 weeks *(C)*, and 4 weeks *(D)* after surgery (n = 3). The top and bottom part of each panel represent haematoxylin and eosin (H&E) staining and fatty acid-binding protein 4 staining, respectively. Magnification, 200×. Yellow and black arrowheads indicate the inflammation and ectopic fat accumulation in the injured muscle, respectively. Black arrows represent fatty acid-binding protein 4 protein expression.

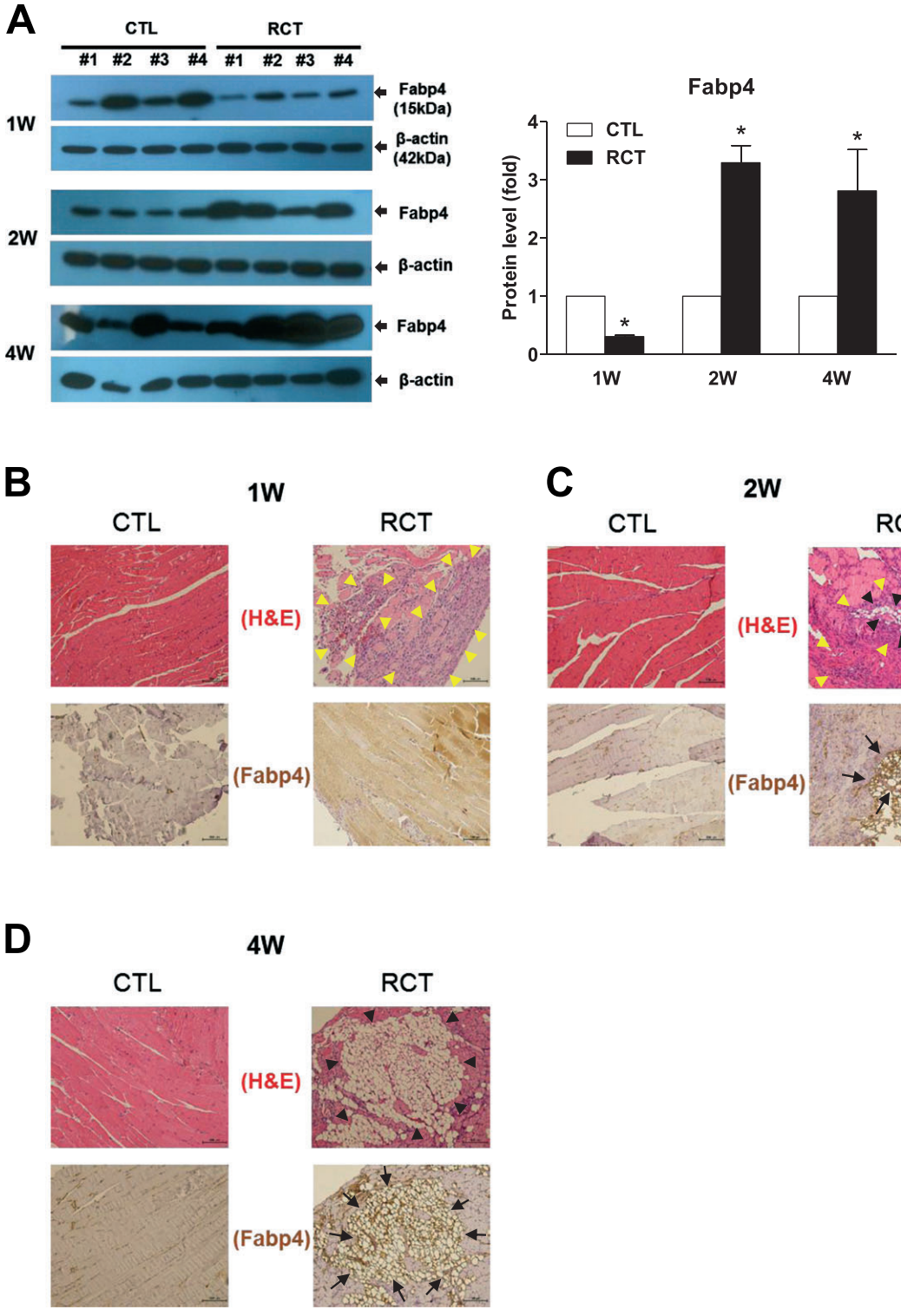

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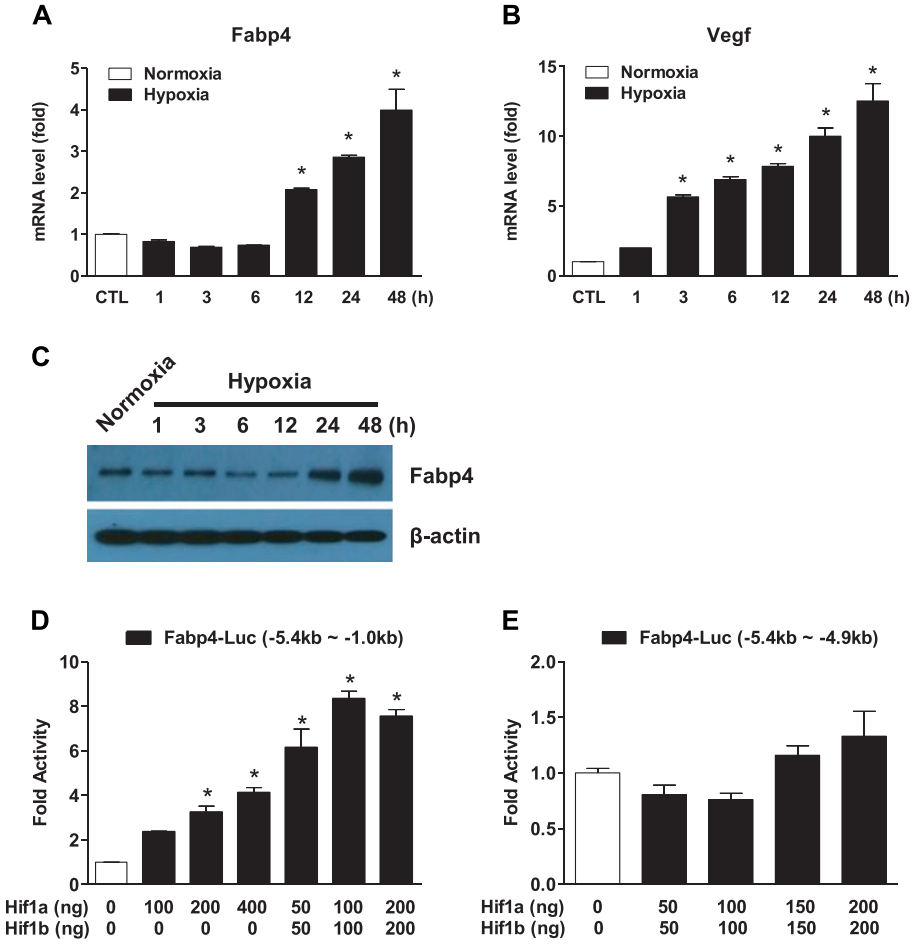
Figure 4 *Fabp4* gene expression is regulated by hypoxia-inducible factor 1 in hypoxic condition. The expression of *Fabp4 (A)* and *Vegf (B)* mRNA in C3H10T1/2 cells after hypoxic challenge for the designated times. *(C)* Fatty acid-binding protein 4 protein expression in C3H10T1/2 cells after hypoxic challenge for the designated times. *(D, E) Fabp4* promoter-driven luciferase activities by overexpression of hypoxia-inducible factor 1α and hypoxia-inducible factor 1β. The *Fabp4*-Luc (−5.4 kb ~ −1.0 kb) reporter construct harbours three hypoxia-inducible factor 1-binding sites, while the *Fabp4*-Luc (−5.4 kb ~ −4.9 kb) reporter construct has no hypoxia-inducible factor 1-binding site. Data represent the mean ± SEM fold increase relative to controls. Differences were considered significant at $P < 0.05$.



A number of studies have reported that muscle fatty infiltration has various causes such as aging, muscle injury, and hormonal changes.[18] Rotator cuff tear is a representative musculoskeletal disorder leading to fatty infiltration in aged individuals. In this study, we utilized a mouse RCT model to investigate the role of FABP4 in muscle fatty infiltration because a previous study demonstrated that a mouse model of RCT shows similar pathological changes, including muscle atrophy and fatty infiltration seen in patients.[9] Interestingly, mice showed a surprisingly fast wound healing after surgery. Thus, for the analysis of characteristics of RCT, we set three time points (1, 2, and 4 weeks). Within 4 weeks after surgery, all mice were nearly recovered from the tendon and muscle injuries. However, they showed pathological responses strongly similar to those observed in previous animal studies on RCT, an inflammatory response in early stage and intramuscular fatty infiltration as well as up-regulation of adipogenic and myogenic gene expression.[8,25,41] Therefore, the mouse RCT model can be considered an appropriate model to understand the many aspects of the rotator cuff response to injury including muscle fatty infiltration within a short experimental period as compared with other animal models such as rabbit, sheep, and primates.

Most studies have asserted that the underlying mechanism of the ectopic fat accumulation is mediated by adipogenic differentiation of muscular stem cells. Uezumi *et al.* reported that PDGFRα-positive mesenchymal progenitors are the major contributor to ectopic fat cell formation in skeletal muscle.[21] Moreover, they suggested that mesenchymal progenitors are the main origin of not only fat accumulation but also fibrosis in skeletal muscle.[22]

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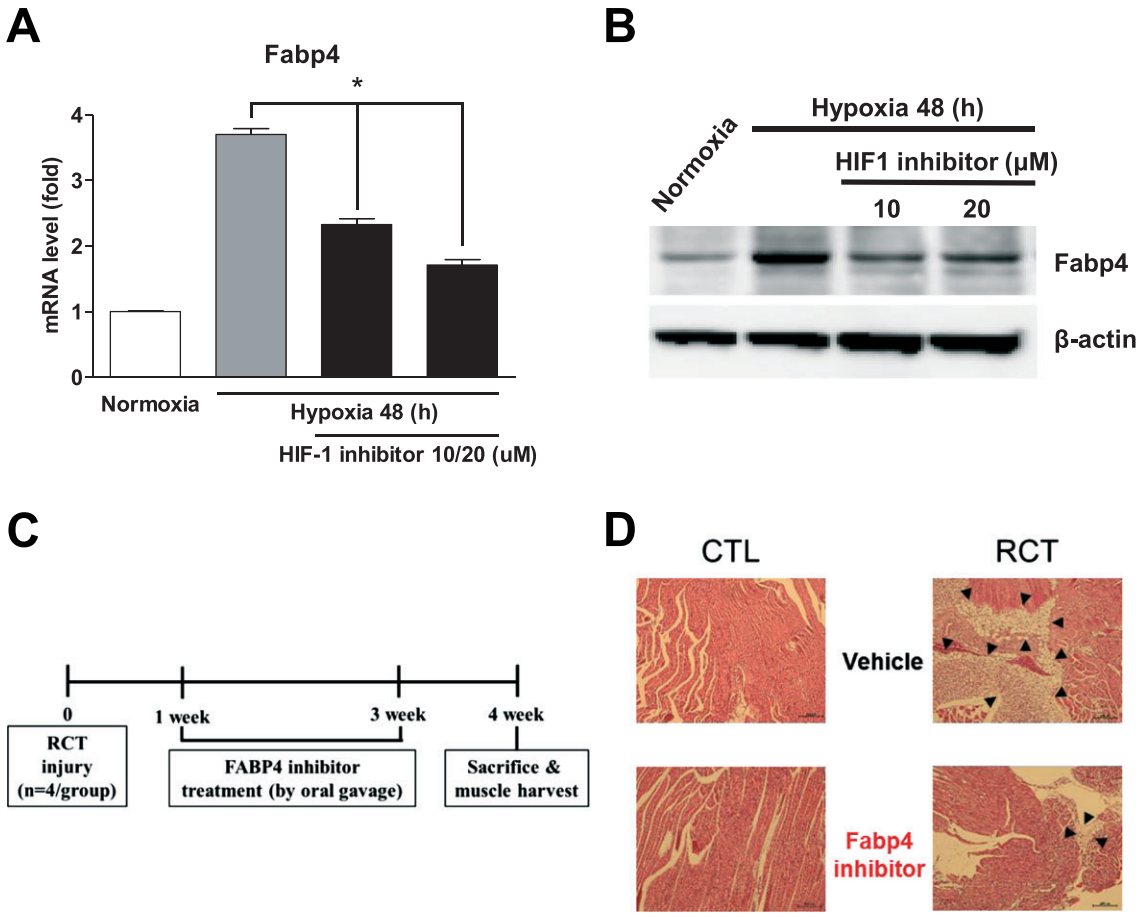
Figure 5 Hypoxia-induced fatty acid-binding protein 4 expression and rotator cuff tear-mediated fat accumulation is reduced by inhibition of hypoxia-inducible factor 1 and fatty acid-binding protein 4, respectively. *(A)* Expression of *Fabp4* mRNA in C3H10T1/2 cells after hypoxic challenge for 48 h in the presence or absence of hypoxia-inducible factor 1 inhibitor. Dimethyl sulfoxide was used as a control. *(B)* Expression of fatty acid-binding protein 4 protein in C3H10T1/2 cells after hypoxic challenge for 48 h in the presence or absence of hypoxia-inducible factor 1 inhibitor. *(C)* Scheme showing the surgical and fatty acid-binding protein 4 inhibitor treatments. One week after rotator cuff tear in mice (n = 4), fatty acid-binding protein 4 inhibitor BMS309403 was administered daily by oral gavage (15 mg/kg) for 2 weeks. Dimethyl sulfoxide was used as a vehicle. One week later (4 weeks after rotator cuff tear), the mice were sacrificed, and isolated muscles were used for H&E staining. *(D)* Comparison of intramuscular fat accumulation after rotator cuff tear in the presence or absence of fatty acid-binding protein 4 inhibitor in mice (n = 4/group). Magnification, 100×. Black arrowheads indicate ectopic fat accumulation in the injured muscle.



Adipogenic transcription factors have been considered key regulators for fatty degeneration of muscle after RCT. One research group reported that the transcription factors PPARγ, C/EBPβ, and Myf-5 play crucial roles in impairment and regeneration of rotator cuff muscles after tendon tears in a sheep model.[8] Joshi *et al.* suggested that mammalian target of rapamycin signaling plays an important role in muscle atrophy after massive RCTs in a rat model and mediates rotator cuff fatty infiltration via SREBP-1 and PPARγ.[23] However, it is doubtful that the population of multipotent stem cells is sufficient in the old individual; stem cell numbers and activities decrease during aging.[42] As another mechanism of fatty infiltration, a previous study suggested that fatty infiltration after tendon release was associated with substantial, irreversible architectural changes in the muscle, such as an increase in the pennation angle and shortening of the muscle fibers.[43] In this way, muscle fatty infiltration may not only depend on adipogenic differentiation of stem cells, but also associate with other unknown mechanisms. Our study suggests such another possible mechanism for muscle fatty infiltration, at least after RCT.

Fatty acid-binding protein 4 has been initially regarded an adipocyte-specific protein, but recent studies suggest that it may be more widely expressed.[44] In our study, mRNA and protein expression of FABP4 were strongly induced by RCT in mice, suggesting that FABP4 has a specific role in muscular events. Surprisingly, gene expression of *Fabp3*, the muscle type of FABP, was not changed by RCT. This result indicates that FABP4 is induced by certain stimulation in

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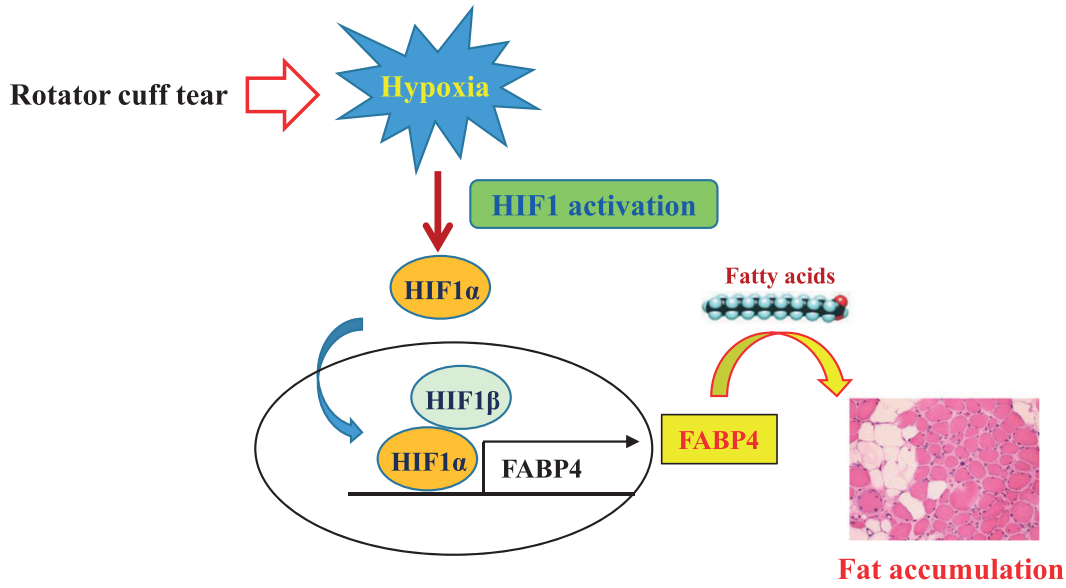
Figure 6 Proposed mechanism for muscle fatty infiltration after rotator cuff tear injury. Rotator cuff tear induces hypoxic condition in the injured area. Hypoxia activates hypoxia-inducible factor 1α in the cytoplasm leading to its translocation to the nucleus. Together with its molecular partner hypoxia-inducible factor 1β, hypoxia-inducible factor 1 complex binds to the promoter of *Fabp4*, which in turn regulates *Fabp4* gene transcription. Fatty acid-binding protein 4 protein binds to fatty acids and causes fat accumulation in the injured muscle.



the muscle, rather than FABP3, which may exist natively as a muscular fat-binding protein. Meanwhile, previous studies have reported that in adipocytes, the DNA-binding protein C/EBP interacts with the FABP4 promoter and elevates FABP4 gene expression,[45] and that pro-adipogenic PPARγ agonists induce FABP4 production and secretion.[46] In accordance with previous studies,[1,8] we observed up-regulation of the master adipogenic transcription factors C/EBPα and PPARγ expression by RCT. Therefore, we cannot rule out the possibility of C/EBPα or PPARγ-mediated FABP4 expression. However, the significant reduction in hypoxia-induced FABP4 expression by HIF1 inhibitor strongly indicates direct regulation of FABP4 expression by HIF1. Moreover, the promoter assay results demonstrated that HIF1-mediated *Fabp4* gene regulation depends on the HIF1 response element in the *Fabp4* promoter. A previous study demonstrated that tendinopathy and RCT are associated with hypoxia and apoptosis.[39] It has been also reported that tendon retraction in full-thickness medium-sized RCT is characterized by neovascularity, increased VEGF/HIF expression, fatty infiltration, and muscle atrophy,[40] showing substantial involvement of HIF1 in RCT. In addition, a recent study demonstrated that FABP3 and FABP7 are induced by HIF-1α and lead to a significant lipid droplet accumulation in hypoxia.[47] Together with our present finding, these evidences indicate that RCT-induced hypoxic condition activates HIF1 and further regulates FABP expression (*Figure* 6).

In conclusion, while adipogenic differentiation of muscle stem cells is considered a main mechanism of muscle fatty infiltration after injury, our study identified another mechanism involving FABP4 expression through the hypoxic condition induced by RCT. The regulation of *Fabp4* gene transcription is mediated by direct binding of HIF1 to the *Fabp4* gene promoter. We confirmed that FABP4-mediated intramuscular fat accumulation was remarkably reduced by FABP4 inhibitor treatment. Taken together, our results suggest that fat accumulation induced by FABP4 may lead to fatty infiltration in the injured muscle by prompt fat migration, differently from the adipogenic transcription factor-dependent pathway in muscle stem cells, which may have a role in the late stage of fatty infiltration. Further studies will need to verify the origins of the lipids mobilized by FABP4 and to identify the differences between adipogenic differentiation-dependent and FABP4-dependent mechanisms of fatty infiltration. It is also important to evaluate the clinical application of FABP4 inhibitors, which are used often as candidate therapeutics for diabetes and atherosclerosis in animal studies, for muscle degeneration or fatty infiltration.

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Conflict of Interest

None declared.

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