Original Article Impaired bone defect and fracture healing in dystrophin/utrophin double-knockout mice and the mechanism

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Abstract: This study investigated the role of muscle damage in bone defect healing using skull and tibial double-defect and tibial fracture models in dystrophin^{-/-}/Utrophin^{-/-} double-knockout (dKO-Hom) mice. The skull and tibia bone defect and fracture healing was monitored using micro-CT, histology, immuchistochemistry and quantitative PCR. We found the skull defect healing is not impaired while the tibial defect healing was delayed at day 7 in the dKO-Hom group compared to wild-type (WT) group as revealed by micro-CT. Mechanistically, the number of osteoclasts and osteoblasts significantly decreased in the defect area in dKO-Hom group compared to WT group on day 21. DKO-Hom mice showed higher mortality after fracture (6/12) and significantly impaired fracture healing compared to the other groups as revealed by the micro-CT parameters of the calluses. Histology showed higher osteoclast number in the calluses of dKO-Hom mice than other groups. Furthermore, dKO-Hom mice showed down-regulation of 15-Pgdh, II-4, Bmp7, and Bmp9 at 10 days after tibia fracture and BMP6 and 7 in the muscle. In conclusion, the long bone defect and fracture healing are impaired in dKO-Hom mice which demonstrated significantly muscle sarcopenia and related with disturbance of osteoclastogenesis and osteoblastogenesis. The impaired tibial fracture healing was associated with down-regulation of several genes in the muscle.

Keywords: Muscular dystrophy, bone defect healing, tibia fracture, dystrophin/utrophin double knockout

Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disease caused by mutations in the dystrophin gene. DMD patients become wheelchair-bound due to muscle wasting. The *mdx* mouse is a model for human DMD. It is caused by a single base mutation in the dystrophin gene [1]. Human DMD patients exhibit higher fracture risk due to osteoporosis caused by glucocorticoids treatment [2]. It has been shown that dystrophin^{-/-} (mdx) mice exhibit lower mineral bone density and biomechanical properties at 21 days, when muscle damage is not very obvious. Lower strength, stiffness, and energy absorption capacity have been observed in mdx femurs [3]. Rufo reported that in 6-month male and female *mdx* mice, the tibial trabecular bone volume/total volume (BV/

TV) and trabecular number (Tb.N). was significantly lower, whereas the trabelular separation (Tb.Sp) was significantly higher than age matched control mice. Serum Receptor activator of nuclear factor kB ligand (RANKL) was decreased whereas interleukin (IL-6) was increased in mdx mice. Similar results have been observed in DMD patients [4]. Additionally, mdx mice at 3 months exhibited delay in tibia fracture healing due to chronic inflammation, and inhibiting inflammation could reverse this delay in bone healing [5]. However, another study showed that young mdx mice exhibit spontaneous healing in a 2-mm calvarial bone defect healing model [6]. mdx mice treated with prednisolone or restricted activity showed a ~7% decrease in bone strength, but stiffness was only found reduced in the mice subjected to restricted activity. Intrinsic bone properties were not affected by either treatment [7].

However, the mdx mouse, a common DMD murine model [1, 8], has a complete loss of dystrophin, but has utrophin compensation and does not recapitulate the severe phenotype of DMD patients and showed nearly normal life span. The dystrophin^{-/-}/utrophin^{-/-} (dKO-Hom) mouse model is a more severe mouse model of DMD than the mdx mouse [9, 10]. It has been shown that dKO-Hom mice exhibit severe muscle damage, including continuous degeneration/regeneration, inflammation and necrosis, ectopic calcification, stem cell depletion, activation of non-myogenic MSCs as well as upregulation of different signaling pathways such as Notch and RhoA [11-15]. We and others have reported that dKO-Hom mice exhibit a spectrum of degenerative changes in their bone, articular cartilage, and intervertebral discs and experience spinal deformities, heterotopic ossification [9, 16]. Furthermore, our recent study showed that osteopenia in bone tissues of dKO-Hom mice occurred as early as 4 weeks after birth. The mechanism of development of osteopenia is related with abnormal osteogenesis and osteoclastogenesis due to change of RANK L level [17]. The aim of this study is to investigate whether the delayed bone defect healing is related to muscle pathology and whether the tibia fracture healing is impaired and potential mechanism.

Materials and methods

Ethics of using animals

The use of animals in this study followed the guidelines of US national Research Counsil's "Guide for the care and use of Laboratory Animals" and the US public health service's "Policy on Humane Care and use of Laboratory Animals" and Guide for the care and use of Laboratory Animals. We also implemented the replacement, reduction and refinement principles. The use of animals followed the protocol (AWC-15-0073) approved by the Animal Welfare Committee of the University of Texas Health Science Center (UThealth).

Animal breeding

Dystrophin^{-/-}/Utrophin^{+/-} (dKO-Het) mice were obtained from our collaborator Dr. Bing Wang's laboratory at the University of Pittsburgh. WT mice were purchased from Jackson Laboratories and bred in parallel with the dKO-Het strain of mice. The four groups of male and female mice used in this study were as follows: C57BI/10J (WT), Dystrophin^{-/-} (*mdx*), dystrophin^{-/-}/Utrophin^{+/-} (dKO-Het), and dystrophin^{-/-}/ Utrophin^{-/-} (dKO-Hom) mice at 4 weeks-old. We used young mice for this study as dKO mice only live 8-12 weeks. The use of animals followed the protocols approved by the Animal Welfare Committee of the UThealth. Mice were housed at standard day/night circles and husbandry and access to water and food *ad libitum*. Full nutrition diet gels were provided for dKO-Hom mice and all mice underwent surgery.

Skull and tibia defect surgery

Male and female WT (N = 12, 7 female and 5 males), mdx (N = 8, 4 females and 4 males), dKO-Het (N = 5, 3 females and 2 males) and dKO-Hom (N = 10, 4 females, 6 males) mice were used at 4 weeks old for skull and tibia double defect surgery. In order to investigate whether muscle force plays a role in bone defect healing of dKO-Hom mice, we introduced non-critical defects in the skull and tibia in the same animal, using 0.9 mm drill burr on the right parietal bone and proximal tibia. The defects were uni-cortical for tibia and full thickness for parietal bone. After surgery, healing of the bone defect was monitored using Viva CT 40 every week for 21 days. At the last time point on day 21, the mice were euthanized after microCT scan, and the skull and tibia were dissected and fixed in 4% neutral buffered formaldehyde (NBF) for 48 hrs for histology.

MicroCT analysis

After the acquisition of 2D images, the 3D overview was analyzed first with consistent dimensions to obtain an overview of bone defect healing in both the skull and the tibia. To quantify the defect healing, the bone growth into the defect area was carefully contoured manually by choosing 50 slices covering the defect area. Parameters of Gauss = 1, Sigma = 0.8 and threshold = 163 were used for all skull sample analysis. Bone volume and density and other parameters were generated automatically by MicroCT software and compared between groups. The nomenclature of bone microarchitecture followed the guideline of American Society of Bone and Mineral Research [18].

Tibia fracture surgery

Male and female WT (N = 8), mdx (N = 8), dKO-Het (N = 8), and dKO-Hom (N = 12) mice at 4 weeks of age were used for this study. Mice were subjected to midshaft tibial fracture according to the literature with modification [19]. Briefly, an incision was made in the front of the tibia and a pilot hole was made on the proximal tibia right below growth plate with 28G1/2 needles. Then a fracture was made with a 0.7-mm drill burr in the midshaft of the tibia. After creating the fracture, 28G1/2 needle was inserted into the intramedullary cavity through the premade pilot hole for internal fixation. After surgery, the mice were scanned with micro-CT on day 1 to confirm fracture and the status of the pin. Bone fracture healing was monitored by micro-CT at day 7 and 14. At day 21, mice were sacrificed and the fractured bones were harvested and fixed in NBF for 48 hrs and scanned using 15-um voxel size. We choose this time point to sacrifice mice because the life span of the dKO-Hom mice is 8 weeks on average. For micro-CT quantification of the calluses grown in the fracture site, on day 7 and 14, 150 slices (30-µm thickness) surrounding the breaking point of fracture were contoured manually to define the view of interest. On day 21, 300 slices (15-µm thickness) were contoured to match the size of the 150 slices taken at day 7 and 14. Parameters of Gauss = 1, Sigma = 0.8, and threshold = 163were used to quantify callus quality and quantity. Total volume represents callus size. Bone volume (BV) represents the new bone formed in the fracture site. Additionally, another set of male and female WT (N = 5), mdx (N = 5), dKO-Het (N = 7), and dKO-Hom (N = 6) mice at 4 weeks of age were subjected to tibia fracture and sacrificed at day 10 post-fracture for extraction of RNA.

Histology

The skull and tibia defect and fractured tissues were decalcified using 10% Ethylenediaminetetraacetic acid disodium dihydrate (EDTA) plus 1% sodium hydroxide for 4 weeks, and tissues were processed, paraffin-embedded, and sectioned into 5-mm sections. H&E staining was performed routinely. Herovici's staining were performed according to published literature to differentiate collagen type I and III [20, 21]. Tartrate-resistant acid phosphatase (TRAP) staining was performed using the 387A Kit (Sigma-Aldrich) following the manufacture's protocol. Immunohistochemistry for osteocalcin was performed using goat anti-osteocalcin (SC-23790 Santa Cruz Biotechnology) in tibial defect tissues, and rabbit anti-osteocalcin (PA5-78870, Thermo Fisher Scientific) was used in tibial fracture tissues due to the discontinuation of the SC 23790 antibody. Biotinylated secondary antibodies used were horse anti-goat IgG-biotin (BA 9500) or horse antirabbit-IgG-biotin (BA-1100, Vector Laboratories). VECTASTAIN® Elite ABC-HRP Kit (PK-6100, Vector Laboratories) was used after treatment with the secondary antibodies, and the DAB kit was used to reveal positive osteoblasts. Hematoxylin QS (H-3404-100, Vector Laboratories) was used for nuclear counterstaining. Images were captured using NIKON CI software. The positive cells and bone area were counted and measured with Image J software and normalized to cells/mm².

Muscle tissues harvesting

In order to identify whether the muscle secretion factors affect bone healing, we harvested thigh muscle tissues from 4 weeks of mice of WT (N = 5), mdx (N = 5), dKO-Het (N = 4) and dKO-Hom (N = 5) mice for RNA extraction. Muscle tissues were carefully dissected to remove fat tissues and placed in Trizol and stored in -80 for RNA extraction as stated below. Semi-quantitative PCR was performed using GoTaq® G2 Hot Start Green Master Mixes (2X) kit from Promega. PCR was done using 20 ul PCR reaction volume, and 1.5% agarose gel was run using 150 V using Bio-Rad Gel system. Q-PCR was performed using SSo Advanced SYBR kit (Bio-Rad).

RNA extraction, cDNA synthesis, and quantitative PCR (qPCR)

In order to investigate the mechanism of impaired fracture healing in dKO-Hom mice, we introduced tibial fracture in mice, which were then sacrificed at 10 days after fracture. Fracture tissues were dissected carefully and any residual muscle and connective tissue was removed. Bone calluses were cut with scissors and then homogenized in 1 ml cold Trizol for 2 minutes. The lysates were centrifuged at 12000 g for 10 minutes at 4°C to remove tis-

sue debris. The supernatant was transferred to another tube for RNA extraction. RNA extraction was performed using Trizol reagent (Invitrogen) following the manufacturer's protocol. One microgram of total RNA was used for cDNA synthesis using the BIO-RAD Iscript Supermix, according to the manufacture's protocol. Subsequently, the synthesized cDNA was diluted 1:5 with DNase/RNase-free water and used as the template for qPCR. A total of 2 µl cDNA per 20 µl reaction was used in qPCR using the Bio-Rad SSo Advanced SYBR kit with the CFX svstem (Bio-Rad). Primers were designed with online software (Primer 3 Input) [22, 23]. Primer information is shown in Supplementary Table 1. We used Delta CT relative to Gapdh expression to represent the gene expression level as it not only reflects the relative expression level, but also the abundance of gene expression in comparison to fold change. If delta CT value the is greater, it indicated the value expression level is lower.

Statistical analysis

All values are expressed in Mean \pm SD unless otherwise stated. Analysis of variance (ANOVA) was used to compare multiple groups followed by Tukey' post-hoc test. Wilcoxon rank sum test was used for higher SD data. A *P*-value <0.05 was considered to be statistically significant.

Results

Tibial defect healing is delayed while skull defect healing is not affected in dKO-Hom mice

All mice with successful surgery and survived until the end of experiments were analyzed. We found no difference in the bone volume (BV) of the bone in-growth in the skull defect in dKO-Hom mice compared to other mice groups. However, the new bone density in the skull defect was significantly lower in dKO-Hom mice than in WT, mdx and dKO-Het mice (Figure 1A-C). Furthermore, the skull parietal bone defects were not healed at 21 days after surgery in any of the groups. In contrast, tibial defect was almost completely healed on day 21 in all groups. Quantification of bone ingrowth in the tibial defect indicated that on day 7 post injury, the new bone volume (BV) in the defect area was significantly lower in dKO-Hom mice than in WT, *mdx*, and dKO-Het mice. At 2 weeks, the BV was significantly lower in dKO-Hom mice than in mdx mice. No statistical differences were found among the other groups. At 3 weeks, no statistical differences in BV were found between dKO-Hom mice and the other groups. However, the density of the newly regenerated bone was significantly lower in dKO-Hom mice than in WT and *mdx* mice at both 14 and 21 days post-injury (**Figure 1A**, **1D**, **1E**).

Fewer osteoclasts and osteoblasts in the defect area during tibial defect healing

Next, we performed Herovici's staining to investigate the formation of major bone matrix collagen type 1 (red color). Our results indicated that the skull defects did not heal with collagen type I bone matrix but with fibrotic tissues (blue) in all the groups (region between black arrows), and there was no apparent difference between dKO-Hom mice and WT, mdx, dKO-Het groups (Figure 2A). In contrast, the tibial defect healed almost completely in all the groups as shown by positive collagen type I matrix staining (red). However, the healed defect exhibited lower density of collagen 1 in the dKO-Hom mice than in the other groups (between yellow arrows) (Figure 2A). Furthermore, H&E staining indicated that the newly regenerated bone area showed trabecular bone like morphology, and dKO-Hom mice demonstrated thinner trabecular bone in the healed defect area (green arrows) (Figure 2B). In addition, we found that the number of TRAP⁺ osteoclasts in the tibial defect area of dKO-Hom mice was significantly lower than that of WT, *mdx*, and dKO-Het mice (Figure 3A, 3C). In addition, we found that the number of osteocalcin⁺ osteoblasts also decreased in the defect area of dKO-Hom mice compared to WT mice (Figure 3B, 3D).

Tibial fracture healing is impaired in dKO-Hom mice compared to mdx, other groups of mice

DKO-Hom mice demonstrated higher mortality after fracture (6/12) with successful surgery and internal fixation placement, whereas no mortality was observed in other groups after bone injury. The microCT results showed visible callus formation in all groups at 7 days, the callus size increased at day 14, and fracture union was observed in all groups at day 21 (**Figure 4A**). Furthermore, segmental analysis of newly formed calluses surrounding the fracture side indicated that the calluses in dKO-Hom mice group were smaller and less dense at day 7, 14, and 21 (**Figure 4B**). Quantification



Figure 1. MicroCT analysis of skull and tibial defects in four groups of mice. A. MicroCT overview of skull and tibial defects at different time points. The skull defect did not heal at three weeks in any of the groups; however, tibia defect showed healing at day 7 and had nearly completely healed at 21 days in the different mouse groups. dKO-Hom mice showed incomplete healing even at day 21 when compared to the other groups. B. Quantification of new bone volume in the skull defect showed no differences between dKO-Hom mice and the other groups. C. Quantification of new bone density showed new bone density was lower in dKO-Hom mice when compared to the other groups. D. Quantification of tibial defect bone volume. dKO-Hom mice showed lower bone volume at day 7 compared to WT, *mdx* and dKO-Het mice. At day 14, the bone volume was lower than that of *mdx* mice. At day 21, no difference was found between dKO-Hom mice at both day 14 and day 21. **P*<0.05, ***P*<0.01, ****P*<0.001.

of the callus demonstrated that the callus size (total volume) was significantly smaller in dKO-Hom mice than in WT, *mdx*, and dKO-Het mice (**Figure 4C**). No statistical difference was observed between *mdx*, dKO-Het, and WT mice. The BV of the callus was also significantly lower in dKO-Hom mice compared to that of WT, mdx and dKO-Het mice. No statistical difference between any other groups at any time points (**Figure 4D**). Furthermore, the BV/TV of callus in dKO-Hom mice at day 7 was also sig-

nificantly lower than that in mdx mice. By day 14 and day 21, the BV/TV was significantly lower in dKO-Hom mice compared to the WT, *mdx* and dKO-Het mice (**Figure 4E**). The trabecular thickness (Tb.Th) of fracture in dKO-Hom mice was also significantly smaller when compared to WT and mdx mice at days 7 and 14. At day 21, the Tb.Th was significantly lower in dKO-Hom mice than any other groups (**Figure 4F**). No statistical differences were found for Tb.N and Tb.Sp (Data not shown).



Figure 2. Histology at day 21 after skull and tibial defect surgery. A. Herovici's staining of 1-mm skull defect. Red color indicates the collagen I matrix of the bone. The area between two black arrows indicates the area of skull defect. Incomplete healing of the bone defect was found in all groups. No differences were found between the groups. The area between two yellow arrows in the tibial defect indicates healing in all groups. However, the healed defect area revealed thinner trabecular bone in dKO-Hom mice than in WT, *mdx* and dKO-Het mice. B. H&E staining of the tibial defect indicated by area between two green arrows.

The impaired tibial fracture healing of dKO-Hom mice was correlated with increased osteoclasts activity

H&E staining showed the fracture calluses in each group were mainly consisted of trabecular bone, the calluses tend to be remodeled to cortical bone in the WT, mdx and dKO-Het mice, but not in dKO-Hom mice group. The calluses in dKO-Hom mice group was smaller (Figure 5A). Herovicis' staining showed the major bone matrix collagen type I is less dense in dKO-Hom mice than that of control mice (Figure 5B). TRAP staining indicated the number of osteoclasts in the tibia fracture site of dKO-Hom mice are significantly higher than that of WT, mdx and dKO-Het mice at 21 days after tibia fracture (Figure 5C, 5E). We found no difference in osteocalcin positive osteoblasts (Figure 5D, 5F).

Impaired tibial fracture healing was associated with down-regulation of BMPs and inflammatory genes

We analyzed 36 genes involved in different pathways that are listed in <u>Supplementary</u>

Table 1. Among the five genes related to osteogenesis and osteoclastogenesis, no statistical differences were found for Runt related transcription factor 2 (Runx2), osterix (Osx), and alkaline phosphatase (Alp) among the different groups. Cathpsin K (CatK) was significantly decreased in dKO-Het mice compared to WT mice. No differences were found between the other two groups. The tartrate resistant acid phosphatase (Trap) expression was significantly decreased in dKO-Hom mice compared to WT control mice, whereas no statistical differences between any other groups (Figure 6A). For genes related to endochondral bone formation-mediated fracture healing, we found that the expression of the mesenchymal stem cell marker platelet derived growth factor receptor alpha (Pdgfra) and cartilage marker collagen type 2 (Col2A1) were relatively higher in dKO-Hom mice than in WT mice (P = 0.1). In contrast, osteocalcin (Ocn) was significantly lower in dKO-Hom mice than in WT mice, and no statistical differences were found for Prx1 and Sox9 among any of the groups (Figure **6B**). We examined inflammatory related genes including cyclooxygenase 2 (Cox2), 15-prostagladin dehydrogenase (15-Pgdh), prostaglan-



Figure 3. Osteoclasts and osteoblasts in the healed tibial defect bone area. A and C. TRAP staining and quantification for osteoclasts. The boxed area highlights TRAP-positive osteoclasts in violet-red color on the bone surface. The numbers of TRAP-positive osteoclasts in *mdx*, dKO-Het, and dKO-Hom mice were significantly lower than that in WT mice. B and D. Immunohistochemistry staining and quantification of osteocalcin to detect osteoblasts. Osteoblasts on the bone surface are stained brown. The boxed area highlights osteocalcin-positive osteoblasts. The number of osteoblasts was significantly lower in dKO-Hom mice than in WT mice. **P*<0.05, ***P*<0.01; Wilcoxon rank sum test. No statistically significant difference was observed among the other groups. Error bars represent standard errors of mean.

dins receptor 2 and 4 (EP2, EP4), tumor necrosis factor stimulated gene 6 (TSG6), CD68, transglutaminase 2 (Tgm2), monocyte chemoattactic protein 1 (Mcp1), interleukin 4 (IL4), we found that the expression levels of 15-Pgdh and IL-4 were significantly lower in dKO-Hom mice compared to WT mice (Figure 6C). Furthermore, the expression of CD68 and Cox2 were significantly lower in dKO-Het mice than in WT mice (Figure 6C). Among the bone morphogenetic proteins (BMP) 2, 3, 4, 6, 7, 9 and their antagonist, noggin, we found that Bmp7 and Bmp9 were significantly lower in dKO-Hom mice group than in the WT mice control group. No statistical differences were found for other genes among any of the other groups (Figure 6D). Finally, we investigated the expression of Wnt signaling pathways molecules including wingless-type-MMTA integration site family 3

(Wnt3A), secreted-frizzled-related protein 1, 3, 4 (sFrp1, sFrp3, sFrp4), β catenin (β -Ctn), and growth factors including vascular endothelial cell growth factor a (Vegfa), hepacyte growth factor (Hgf), insulin-like growth factor 1, 2 (lgf1, lgf2), fibroblast growth factor 2 (Fgf2), and transforming growth factor β 1 (Tgf β 1), we found that sFrp1 was significantly decreased in dKO-Het mice compared to WT mice. No statistically significant differences were found between any other two groups of mice (**Figure 6E**).

Impaired fracture healing correlated abnormal paracrine factors from the dystrophic muscle tissues

We further performed semi-quantitative PCR analysis of osteogenesis and osteoclastogene-



Figure 4. Micro-CT analysis of tibial fracture healing at different time points post-fracture (PF). A. Micro-CT overview of the fracture healing process. Calluses began to form at day 7 PF, and the fracture was mostly healed by day 14 PF, and almost completely healed by day 21 PF. B. Micro-CT images of calluses revealed the newly formed bone sur-

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rounding the break point of fracture site in each group. C. Quantification of the total volume (calluses) demonstrated that dKO-Hom mice formed significantly smaller calluses compared to WT, mdx, and dKO-Het mice. D. The newly formed bony calluses (bone volume, BV) were significantly smaller in dKO-Hom mice than in WT, mdx, and dKO-Het mice. E. The BV/TV in dKO-Hom mice was significantly lower than that in WT, mdx, and dKO-Het mice at days 14 and 21 PF, and significantly lower than mdx mice at day 7 PF. F. Tb.Th was significantly lower in dKO-Hom mice than in WT and mdx mice at days 7 and 14 PF, and significantly lower in dKO-Hom mice than in all other groups of mice at day 21 PF. *P<0.05, **P<0.01, ***P<0.001.



Figure 5. Histology of the tibial fracture callus at day 21. A. H&E staining revealed the overall structure of the callus. Newly formed calluses were composed mainly of trabecular bone and bone marrow. The trabecular bone was smaller in dKO-Hom mice than in WT, *mdx*, and dKO-Het mice. B. Herovici's staining showed the major bone matrix collagen type 1 (red) and collagen type 3 (blue fiber-like structure). All mouse groups showed collagen type 1-positive bony calluses. The collagen type 1 content in dKO-Hom mice appeared less dense than that in WT mice. C. Representative images of TRAP staining for osteoclasts. Insets highlight TRAP⁺ osteoclasts in the boxed area of each 200× image. D. Representative image of osteocalcin immunohistochemistry staining. E. Quantification of osteoclast number in the fracture area. F. Quantification of the osteocalcin⁺ osteobalsts in the defect area. **P*<0.05, ***P*<0.01, ***P*<0.001.



Figure 6. Ouantitative-PCR (gPCR) of tibial fracture calluses at 10 days post-fracture (PF). A. Osteogenesis- and osteoclastogenesis-related genes. No significant differences were observed in expression levels for Runx2, Osx, and Alp between the groups. Catk expression in dKO-Het mice was decreased (higher delta CT value). Trap expression was significantly lower in dKO-Hom mice compared to WT mice, and no differences were found in the other two groups relative to WT. B. Quantification of the expression of endochondral bone formation-related genes. We found expression levels of Pdgfra and Col2A1 were relatively higher (lower delta CT value) in dKO-Hom mice, though not significantly (P = 0.1). The expression of Col2A1 was also significantly higher in mdx mice. On the contrary, the expression of the osteogenic differentiation marker gene Ocn was significantly lower in dKO-Hom mice compared to WT mice. C. Pro- and anti-inflammatory gene expression levels. The levels of CD68 and Cox-2 were significantly lower in dKO-Het mice, and 15-Pgdh and IL-4 levels were significantly lower in dKO-Hom mice, compared to WT mice. No further significant differences were found in the levels of inflammatory markers among any of the groups. D. Expression of

genes encoding bone growth factors and inhibitors. The expression levels of Bmp7 and Bmp9 were significantly lower in dKO-Hom mice than in WT mice. No significant differences were found in the other two groups. Furthermore, no significant differences were observed among any of the mouse groups in the expression of Bmp2, Bmp4, and Postn, or in the expression of the bone formation inhibitory genes Bmp3 and Nog. E. The expression of growth factor and Wnt signaling pathway genes. SFrp1 was significantly decreased in dKO-Het mice compared to WT mice; no significant differences were found for other genes among any of the groups. *P<0.05, **P<0.01 Wilcoxon rank sum test.

sis genes expression in the thigh muscle tissues. We found BMP2, 3, 4, 6, 7 were significantly decreased in the muscle tissues of mdx, dKO-Het and dKO-Hom mice than group. No differences WT were found for Noggin and BMP9. Furthermore, Runx2, CatK and Trap were significantly increased in the muscle tissues of mdx, dKO-Het and dKO-Hom mice compared to WT mice. Alp was significantly decreased in the muscle tissues of mdx, dKO-Het and dKO-hom mice compared to WT mice (Figure 7A, 7B). Furthermore, we performed quantitative PCR analysis for the selected the differential expressed genes observed in semi-quantitative PCR, we found BMP6 and BMP7 were significantly lower in the *mdx*, dKO-Het, and dKO-Hom mice than WT mice (Figure 7C). Runx2, Osx, CatK, and Trap were significantly higher in the mdx, dKO-Het and dKO-Hom mice than WT mice, with dKO-Hom mice has the highest expression level (Figure 7D).



Figure 7. Semi-quantitative and q-RT-PCR results for the muscle tissues of 4 weeks-old mouse. A. Electrophoresis results of semi-quantitative-PCR of osteogenic and osteoclastogenesis related genes. The PCR products gel bands were cropped from full-length gels and arrange together to save space. Each gel was run on individual gel due to the limitation of the size of the gel box. All gels were imaged with the same exposure time. B. The relative expression level of genes by band intensity quantification. *P<0.05, **P<0.01, ***P<0.001. C. Q-PCR analysis of differential expressed BMP genes from semi-quantitative PCR. *P<0.05. D. Q-PCR results of osteogenesis and osteoclastogenesis markers. *P<0.05, **P<0.01, ***P<0.001.

Discussion

In this study, we found that dKO-Hom mice exhibited delayed tibial defect healing, but not skull defect healing, compared to WT mice. The numbers of osteoclasts and osteoblasts both decreased in the tibial defect in dKO-Hom mice. Moreover, we found that tibial fracture healing of dKO-Hom mice was significantly impaired compared to WT, *mdx* and dKO-Het mice. The impaired fracture healing was related to a higher number of osteoclasts at the fracture site. The death rate for dKO-Hom mice after tibial fracture was high (6/12), while all the animal survived the bone injury in the other groups. Impaired fracture healing was found to be associated with lower levels of Bmp7, 9, 15-Pgdh, IL-4 in the calluses and lower BMP6, 7 in the muscle tissues.

Previously we have shown that dKO-Hom mice exhibited impairment in tibial defect healing [16], however, whether this delayed defect healing is related to muscle-bone interaction and abnormal paracrine factors in the muscle is still unknown. Because parietal bone has no muscle attachment whereas the tibia has abundant muscles, we tested this hypothesis by creating defects of the same size in the right parietal bone and the proximal tibia (anterior). We found that a 1-mm skull defect did not heal in any of the groups including the WT control mice, and the dKO-Hom mice did not show any decrease in skull defect healing, while the tibial defect healing was significantly delayed, when compared to the other groups. This indicated that muscle pathology likely plays a role in the healing of bone defects as dKO-Hom

mice showed most severe muscle pathology and sarcopenia compared to *mdx* and dKO-Het mice. Indeed, our q-PCR results showed dystrophic muscle tissues expressed lower level of BMP6 and BMP7 and higher level of osteoblasts and osteoclasts marker. These abnormal signaling pathways maybe related with the large amount of heterotopic bone formation in the muscle tissues of *mdx*, dKO-Het and dKO-Hom mice we observed previously [17].

In order to investigate the mechanism of impaired tibial defect healing, we looked at osteoblast and osteoclast activity during defect healing. We found the osteoblast number was decreased in the healed defect area of dKO-Hom mice than WT mice. We also found that, at day 21, in the healing process, the osteoclast number was decreased in the defect area in dKO-Hom mice when compared to the other groups. However, in tibial fracture callus, we found higher numbers of TRAP⁺ osteoclasts in dKO-Hom mice than in other groups at day 21. This discrepancy might be attributed to different pathological process at different ages as osteoclast activity undergoes dynamic changes.

Furthermore, in this study, we found that tibial fracture healing in dKO-Hom mice is impaired compared to WT mice, whereas mdx and dKO-Het mice did not show impaired fracture healing. The high mortality rate of dKO-Hom mice likely were due to the short life span of the mice, as the untreated mice died at 6-12 weeks [9]. Previously, it has been shown that 6-month-old mdx mice demonstrated delayed fracture healing at day 7 and day 14, and even formed bigger callus and more bone at day 21 post-fracture. The difference between our results and those in this report might be attributed to the difference in the age and the histopathology of the mice used; the discrepancy might also have arisen because we used internal fixation whereas the other study used nonfix tibia fracture model [5]. To further investigate the mechanism of impaired fracture healing, we performed qPCR analysis of the mRNA expression of 36 genes. Among these, we found that 15-Pgdh was decreased in dKO-Hom mice, 15-Pgdh is an enzyme that catalyzes the degradation of prostaglandins produced by cyclooxygenase 1 and 2. It has been shown inhibition of 15-Pgdh enhanced liver regeneration and bone marrow recovery after radiation [24]. Cox-2 was not increased at the fracture site in dKO-Hom mice; therefore, decrease of 15-Pgdh likely increased tissue prostaglandin E2 (PGE2) signaling transduction. PGE2 is very important for bone formation during fracture healing, and a decrease in PGE2 level caused by Cox-2 knockout resulted in delayed fracture healing in mice [25]. Cox-2 knockout musclederived stem cells also showed impaired bone regeneration [21]. However, persistently higher PGE2 levels caused by downregulation of 15-Pgdh may have a detrimental effect, as PGE2 is also a mediator of inflammation. Indeed, a previous study has shown that 6-month-old mdx mice also showed delayed fracture healing due to chronic inflammation [5].

Furthermore, we found that IL-4 expression is significantly reduced in dKO-Hom mice. It has been shown that macrophages play an important role in endochondral bone fracture healing. Application of IL4 and 13 induced the M2 macrophage phenotype and enhanced fracture healing [26]. However, IL4/IL13 knockout mice did not show impaired fracture healing compared to control mice [27]. Therefore, decreased IL-4 may cause delayed M2 macrophage polarization as well as fracture healing. However, we did not observe differences between dKO-Hom and WT mice in the M2 macrophage (transglutaminase 2, TGM2) at day 10 post-fracture. Therefore, other mechanism may exist.

Our results also demonstrated that Bmp7 and Bmp9 were downregulated in fracture calluses in dKO-Hom mice compared to WT control mice. Both BMP7 and BMP9 are important bone growth factors. BMP7 together with BMP2 are the two recombinant proteins that have been approved by the US Food and Drug Administration, and have been extensively studied to promote bone accrual in osteoporosis and repair fracture [28-31]. BMP9 protein and adenovirus-delivered BMP9 have been shown to effectively promote osteogenesis of bone marrow mesenchymal stem cells, as well as bone regeneration, bone defect healing, and fracture healing in vivo [32-35]. Furthermore, the muscle tissues also expressed lower level of BMP6 and BMP7. Therefore, down-regulation of Bmp7 and Bmp9 in the fracture site might be responsible for the delayed fracture healing in dKO-Hom mice. Surprisingly, the Rux-2, Osx, CatK and Trap were all significantly increased in the muscle tissues. These ectopic expression of osteoblastogenesis and osteoclastogenesis related genes were most likely correlated with the highly heterotopic bone formation in the muscle tissues we demonstrated previously [17]. The abnormal activation of osteogenesis and osteoclastogenesis genes likely contributed to the impaired fracture healing in the tibia

as muscle and bone is closely interacted in the long bone.

In conclusion, we revealed that dKO-Hom mice showed delayed bone defect healing in the tibia, but no delay in skull defect. The delayed defect healing was associated with decreased osteoclasts and osteoblasts. Moreover, tibial fracture healing was also impaired in dKO-Hom mice which correlated to the lower expression of IL-4, 15-Pgdh, Bmp7, and Bmp9 during the healing process and lower Bmp6 and Bmp7 in the muscle tissues. This study implied that high fracture risk in patients with DMD may not be the sole cause of osteoporosis due to glucocorticoid intake commonly prescribed in clinic.

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Disclosure of conflict of interest

None.

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Gene ID	Accession#	Forward Primers (5'-3')	Reverse primers (5'-3')	Product size (bp*)
Runx2	NM_001146038	cccagccacctttacctaca	tatggagtgctgctggtctg	150
CatK	NM_007802.4	ccagtgggagctatggaaga	tggttcatggccagttcata	159
CD68	BC021637.1	ttctgctgtggaaatgcaag	agaggggctggtaggttgat	241
Tgm2	BC016492.1	gccaaccacctgaacaaact	cagggtcaggttgatgtcct	225
Wnt3A	M32502.1	ggtggagaaacaccgtgagt	aaagttgggggagttctcgt	115
Tgfβ1	NM_011577.1	tgagtggctgtcttttgacg	tctctgtggagctgaagcaa	292
Cox-2	NM_011198.3	gggcccttcctcccgtagca	ccatggcccagtcctcgggt	232
Ep2	BC005440.1	atgeteetgetgettategt	agggcctcttaggctactgc	126
Ep4	BC011193.1	tggctgtcactgaccttctg	tgcatagatggcgaagagtg	254
15-Pgdh	AK146038.1	aggtagcattggtggattgg	ccacatcacactggacgaac	105
Bmp2	BC100344.1	tgcaccaagatgaacacagc	gtgccacgatccagtcattc	201
Bmp3	BC117749.2	tgctcccatcacactcagag	ctcgtgtgctctggaggtaa	222
Bmp4	BC052846.1	ggttcctggacacctcatca	ctgattctgacatgctggcc	195
Bmp6	BC138593.1	tctacaacgccctgtccaat	tgtcgttgatgtggggggagaa	283
Bmp7	BC010771.1	ataattcggcgcccatgttc	ggttgacgaagctcatgacc	186
Bmp9	AF188286.1	gacagtgagacttgggacca	ctggtctccttggtcccatt	275
Osx	AF184902	actcatccctatggctcgtg	ggtagggagctgggttaagg	238
lgf2	NM_010514.3	gtcgatgttggtgcttctca	aagcagcactcttccacgat	185
Vegfa	NM_001025250.2	cgccgcaggagacaaaccgat	accogtocatgagotoggot	151
Fgf2	NM_008006.2	agcggctctactgcaagaac	gccgtccatcttccttcata	183
Sox-9	NM_011448.3	cggacaagcggaggccgaag	cgtcgcggaagtcgatgggg	276
TRAP	BC012911.1	cagcagccaaggaggactac	acatagcccacaccgttctc	190
Mcp1	NM_011333.3	cacagttgccggctggagca	ttgggacacctgctgctggt	147
Pgdfrα	BC053036.1	tggcatgatggtcgattcta	cgctgaggtggtagaaggag	152
Col2a1	BC051383.1	gccaagacctgaaactctgc	gccatagctgaagtggaagc	239
β-Catenin	M90364.1	acagggtgctattccacgac	ctgcacaaacaatggaatgg	217
Osteocalcin	U11542.1	aagcaggagggcaataaggt	tttgtaggcggtcttcaagc	156
Periostin	BC031449.1	aaccaaggacctgaaacacg	gtgtcaggacacggtcaatg	170
sFrp3	U88568.1	caagggacaccgtcaatctt	cgatccttccacttctcagc	151
Alp	BC065175	gctgatcattcccacgtttt	ctgggcctggtagttgttgt	204
Tsg6	NM_009398.2	cggataccccattgtgaaac	tcctttgcatgtgggttgta	126
Prx1	BC092372.1	gcggagaaacaggacaacat	acttggctcttcggttctga	164
lgf1	NM_010512.5	tggatgctcttcagttcgtg	gcaacactcatccacaatgc	173
Hgf	BC119228.1	ttcccagctggtctatggtc	tggtgctgactgcatttctc	237
sFrp1	U88566.1	cgagtttgcactgaggatga	cgttcttcaggaacagcaca	143
sFrp4	AF117709.1	gagtggcgttcaaggatgat	ttgcgcttttcttcaggttt	274
Noggin	BC138148.1	atccaagtctgtgcacctca	acactcggaaatgatggggt	103
114	AF352783.1	tcaacccccagctagttgtc	tgttcttcgttgctgtgagg	177
Gapdh	BC145812.1	ccggggctggcattgctctc	gtgttggggggccgagttggg	208

Supplementary Table 1. Primers information

*bp, base pair.