Influence of Bone and Muscle Injuries on the Osteogenic Potential of Muscle Progenitors: Contribution of Tissue Environment to Heterotopic Ossification

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ABSTRACT

By using surgical mouse models, this study investigated how the tissue environment influences the osteogenic potential of muscle progenitors (m-progenitors) and potentially contributes to heterotopic ossification (HO). Injury was induced by clamping the gluteus maximus and medius (group M) or osteotomy of greater trochanter (group O) on the right hip, as well as combined muscle injury and osteotomy of greater trochanter (group M+O). The gluteus maximus and medius of the operated hips were harvested at days 1, 3, 5, and 10 for isolation of m-progenitors. The cells were cultured in an osteogenic medium for 3 weeks, and osteogenesis was evaluated by matrix mineralization and the expression of osteogenesis-related genes. The expression of type I collagen, RUNX2 (runt-related transcription factor 2), and osteocalcin by the m-progenitors of group M+O was significantly increased, compared with groups M and O. Osteogenic m-progenitors in group O increased the expression of bone morphogenetic protein 2 and also bone morphogenetic protein antagonist differential screening-selected gene aberrative in neuroblastoma. On histology, there was calcium deposition mostly in the muscles of group M+O harvested at day 10. CD56, representing myogenic progenitors, was highly expressed in the m-progenitors isolated from group M (day 10), but m-progenitors of group M+O (day 10) exhibited the highest expression of platelet-derived growth factor receptor α (PDGFR-α), a marker of muscle-derived mesenchymal stem cells (M-MSCs). The expressions of PDGFR-α and RUNX2 were colocalized in osteogenic m-progenitors.

The data indicate that the tissue environment simulated in the M+O model is a favorable condition for HO formation. Most likely, M-MSCs, rather than myogenic progenitors, in the m-progenitors participate in HO formation.

SIGNIFICANCE

The prevalence of traumatic heterotopic ossification (HO) is high in war injury. The pathogenesis of HO is still unknown. This study clarified the contribution of a tissue environment created by bone or muscle injury to the formation of HO. The study also found that muscle-derived mesenchymal stem cells, but not myogenic progenitors, are involved in the formation of HO. The findings of this study could be used to strategize the prevention and treatment of HO.

INTRODUCTION

Heterotopic ossification (HO) is a pathological condition in which soft tissues, such as muscles, calcify. There are several forms of HO, and traumatic HO appears as a complication of injury, burn, brain injury, or surgery [1, 2]. The prevalence of traumatic HO in a war injury to the extremities is as high as 64% [3, 4]. HO can be painful and impair the motion and function of the affected extremities [5]. Prophylactic radiation and medications such as nonsteroidal anti-inflammatory drugs are effective [6, 7], but these measures are not without serious side effects and may be impractical for severe wartime injuries.

Effective treatments and prevention must rely on the understanding of HO pathogenesis. Although the precise pathogenesis of HO is still unclear, a few clinical conditions have been linked to the development of HO. Analysis of clinical data has concluded that, after fracture or osteotomy, spreading bone marrow to the surgical field is a risk or predisposing factor of HO [8]. This is supported by an animal study, in which HO was successfully induced in rabbits by surgical reaming of femoral medullary canal and intentionally leaving bone
debris and marrow materials in the wound [9]. Bone marrow is well known to contain mesenchymal stem cells (MSCs) and other osteogenic progenitors [10]. It has been suggested that aberrant osteogenic differentiation of bone marrow MSCs/progenitors within skeletal muscle produces HO. MSCs, however, reside in almost all the tissues, including muscles, for homeostasis and repair.

Muscle has outstanding regenerative capacity and maintains a large pool of progenitor cells (m-progenitors). m-Progenitors are heterogeneous, including uncommitted stem cells and committed progenitors [11]. They may reside around muscle fibers or in the interstitial tissue, with a presumed function of myogenesis or angiogenesis, respectively [12, 13]. The myogenic progenitors, commonly known as satellite cells, are responsible for muscle regeneration and can be identified by expression of CD56 [14, 15]. Muscle-derived MSCs (M-MSCs) do not express the myogenic marker Pax7, but can be induced to myogenic differentiation in vitro and contribute to skeletal muscle regeneration in vivo [12]. M-MSCs can be enriched by the expression of platelet-derived growth factor receptor α (PDGFR-α) [14, 16, 17]. Both the myogenic progenitors and M-MSCs are capable of multilineage differentiation, including osteogenesis [14, 18–20].

Tissue environment is critical for the development of HO in muscles. In a classic experimental model of osteoinduction, bone morphogenetic protein (BMP) in the form of demineralized bone matrix was implanted into muscles and induced ossification [21, 22]. Indeed, myogenic progenitors rely on the presence of BMP-2 for robust osteogenic differentiation in vivo and bone formation in vivo [23]. Fracture, osteotomy, and amputation, which naturally initiate osteogenesis for bone healing, are frequently associated with HO. During fracture healing, a featured tissue condition is the upregulation of BMP-2, -6, and -7 in the surrounding tissues, which are largely skeletal muscles [24]. The BMP signaling pathway is also implemented or regulated by BMP antagonists, which is a group of diverse molecules inhibiting BMP signals [25]. For example, noggin and DAN (differential screening-selected gene aberrative in neuroblastoma) can block BMP signals by binding to BMP extracellularly.

Clinical data of hip arthroplasty have shown that muscle injury is a risk factor of HO formation [1, 26]. It is possible that muscle injury might have provided a molecular environment priming the cells for HO formation. To repair the injured muscles, m-progenitors are activated. Under certain conditions, however, m-progenitors can be osteogenic [14, 19]. In the injured muscle, the expression of BMP receptors is upregulated and the phosphorylation of Smad-1, -5, and -8 is increased [27]. BMP receptors and Smad are elements of the intracellular BMP signaling pathway that controls tissue ossification [21].

In this study, injury of gluteus maximus and medius and osteotomy of greater trochanter were created separately and in combination in mice. The three surgical models were used to differentiate the effect of muscle injury and bone fracture on the osteogenic potential of m-progenitors. In each animal model, m-progenitors were isolated from the gluteus maximus and medius and induced for osteogenic differentiation for analysis of their osteogenic potential.

**MATERIALS AND METHODS**

**Surgical Procedures**

A total of 90 C57BL/6 mice (Charles River Laboratories, Frederick, MD, http://www.criver.com), male, 8 weeks of age, were used for this study (approved by MedStar Health Research Institute Animal Care and Usage Committee). The mice were anesthetized with intraperitoneal injection of a cocktail (ketamine 80 mg/kg and xylazine 10 mg/kg). After skin preparation, an incision was made over the right hip to expose the gluteus maximus and medius. Each animal was randomly selected to receive one of the following three surgical procedures: (a) For muscle injury (group M), the gluteus maximus and medius were gently dissected about 5 mm from greater trochanter. Two pairs of hemostatic forceps were used to pinch the muscles in parallel (2–3 mm apart). To be consistent in muscle damage, the hemostatic forceps were clamped two steps at the same time for 5 minutes. (b) For osteotomy (group O), a powered bur was used to cut the greater trochanter, to which the gluteus maximus and medius attach, from the junction with the femoral shaft. The greater trochanter was then loosely reattached with a stitch (Vicryl 5.0). (c) For muscle injury and osteotomy (group M+O), after muscle injury of the gluteus maximus and medius was performed as described for group M, osteotomy of the greater trochanter was conducted as mentioned in group O.

For all the operated mice, the wound was closed in layers, and the skin was approximated with continuous subcutaneous sutures. The animals were not immobilized. Analgesia and antibiotics were given for the first 3 and 7 days after surgery, respectively. In each study group, three to eight mice were sacrificed at days 1, 3, 5, and 10.

On the day of sacrifice, the gluteus maximus and medius on the operated hips were dissected with aseptic techniques and kept in phosphate-buffered saline (PBS). Small portions of gluteus maximus and medius in groups M, O, and M+O, in the operated hips as well as the nonoperated hips, harvested at day 10 were fixed with 4% paraformaldehyde. After processing in 25% sucrose, the tissue samples were embedded in optimal cutting temperature medium (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com) and sectioned with a cryostat. Tissue sections were stained with von Kossa stain for calcium deposition.

**Isolation of m-Progenitors**

Isolation of m-progenitors followed published protocols [28, 29]. Briefly, the harvested gluteus maximus and medius from each mouse were minced and digested in 1% collagenase (type I; Thermo Fisher Scientific Life Sciences) for 2 hours at 37°C on a shaker. The digest was filtered through a 70-μm cell strainer. The collected cells were washed three times with PBS. After cell counting, the isolated cells were plated at a density of 3,000 cells per cm² and cultured with Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific Life Sciences), supplemented with 10% fetal bovine serum, with 5% carbon dioxide in the air at 37°C. The cells were passaged at 70% confluence and used at passage 3 for this study. At each passage, cells isolated from normal gluteus maximus and medius were sampled (n = 3) for flow cytometry of CD56, PDGFR-α, CD31, and CD34 to track the changes of cell subpopulations (supplemental online Fig. 1).

**Flow Cytometry for the Expression of MSC Markers**

According to the recommendation of International Society for Cellular Therapy, CD73, CD90, and CD105 are the common MSC cell surface markers [30]. m-Progenitors isolated from the gluteus maximus and medius that were harvested on day 10 in the groups...
M, O, and M+O were incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies of CD73, CD90, and CD105 (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). FITC-conjugated normal mouse IgG1-γ (BD Biosciences) was used as an isotype control. Propidium iodide was used for exclusion of dead cells. Flow cytometry was also performed on myogenic progenitor marker CD56 (phycoerythrin-conjugated antibody; Abcam, Cambridge, MA, http://www.abcam.com) and M-MSC marker PDGFR-α (allophycocyanin-conjugated antibody; Abcam). The resulting data were analyzed with FlowJo software (Tree Star, Ashland, OR, http://www.flowjo.com). During data analysis, the same gate was used for the cells of three study groups and the isotype control.

Osteogenic Differentiation of m-Progenitors

At passage 3, m-progenitors were plated in 48-well plates and cultured in an osteogenic medium, which was based on DMEM and supplemented with 10% fetal bovine serum, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbate [31], for 3 weeks. m-Progenitors were also cultured in regular tissue culture medium for experimental controls. In each group and at each time point, m-progenitors from three animals were plated. m-Progenitors isolated from the same animal were plated in duplicate. The medium was changed twice a week. After 3 weeks, a portion of the cultures were fixed with 4% paraformaldehyde and stained with Alizarin red. The staining was viewed under a microscope.

Alizarin red stain of matrix mineralization, representing osteogenesis, was also quantified using a kit (Osteogenesis Quantification Kit, EMD Millipore, Billerica, MA, http://www.millipore.com). Briefly, after Alizarin red staining, 10% acetic acid was added into each well of tissue culture plates and incubated for 30 minutes. Cells and acetic acid were collected and heated to 85°C for 10 minutes. After being cooled on ice for 5 minutes, the slurry was centrifuged at 20,000g for 15 minutes. The supernatant (400 μl) was transferred to a new tube, and the pH was neutralized within the range of 4.1–4.5. Samples (150 μl) and standards were added to an opaque-walled, transparent-bottom 96-well plate and read with a microplate reader at optical density 405 nm.

Quantification of the Expression of Osteogenic Genes With Quantitative Polymerase Chain Reaction

m-Progenitors cultured in osteogenic and control medium for 3 weeks were collected for RNA isolation. RNA was extracted by using the TRIzol method (Thermo Fisher Scientific Life Sciences). Total RNA (100 ng) was reverse-transcribed by using the iScript Reverse Transcription Supermix for reverse-transcription quantitative polymerase chain reaction (qPCR; Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com) with both oligo-dT and random hexamers as primers. First-strand cDNA was stored at −20°C. qPCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). Using SSO Advance Sybr green PCR Master Mix reagents (Bio-Rad Laboratories), each reaction mixture consisted of 12.5 μl of SYBR green PCR reagent, 2.9 μl of first-strand cDNA, optimized volume of 5 mM primers, and diethylphosphocarbonate-treated water, for a total volume of 20 μl. Non-reverse-transcribed samples and reactions without cDNA templates were both included in each PCR plate as negative controls. The 18S ribosomal RNA and β-actin were used as internal standards. After 30 seconds at 95°C, the PCR amplification was performed for 40 cycles; each cycle consisted of amplification at 95°C for 10 seconds and at a primer-specific annealing temperature for 30 seconds. Amplification efficiency of >90% was required for further processing of the data. Each reaction was performed in triplicate. The cycle at which the fluorescent level was statistically above the background was defined as the threshold cycle (Ct). The Ct values of the gene under investigation were normalized against 18S or β-actin expression. The expression of the gene was calculated as $2^{-\Delta\Delta Ct}$ (Real-Time PCR Application Guide, Bio-Rad Laboratories). Osteogenic genes included type I collagen, osteocalcin, and Runx2. In addition, the expression of BMP2, BMP receptor 1A (BMPR1A), noggin, DAN, and alkaline phosphatase (ALP) was performed on osteogenically differentiated m-progenitors isolated from the muscles of groups M, O, and M+O harvested at day 10. Primers (Table 1) were supplied by Integrated DNA Technologies (Coralville, IA, https://www.idtdna.com).

Analysis of Osteogenic Subpopulations in m-Progenitors

m-Progenitors isolated from group M+O at day 10 were cultured for osteogenic induction as previously described. A mouse pre-osteoblast cell line (MC3T3-E1; ATCC, Manassas, VA, http://www.atcc.org) was cultured in an osteogenic differentiation medium containing ascorbic acid. Osteogenesis of MC3T3 cells and m-progenitors was confirmed by Alizarin red staining. The cells were permeabilized with 100% methanol and incubated with a cocktail of antibodies, including RUNX2 antibody conjugated with FITC (Abcam), and antibodies of CD56 and PDGFR-α described previously. MC3T3-E1 cells were used as a positive control of RUNX2 staining. Normal mouse IgG was used as an isotype control as previously described. Flow cytometry was performed, and the data were analyzed for coexpression of RUNX2 with either CD56 or PDGFR-α.

Statistical Analysis

Data from flow cytometry, Alizarin red quantification, and qPCR were expressed as mean ± SD. The cell surface marker expression and osteogenic capacity of m-progenitors among groups M, O, and M+O, at different time points were comparatively analyzed with one-way/two-way analysis of variance (ANOVA), followed with post hoc Tukey’s test. Significance was set as $p < .05$.

RESULTS

Histology

The tissue samples of gluteus maximus and medius from group O harvested on day 10 appeared normal structurally, but samples of groups M and M+O showed severe damage and necrosis (Fig. 1). Calcium deposition was mostly observed in the muscles of the M+O model.

The Cell Surface Marker Profiles of m-Progenitors in Groups M, O, and M+O

For the essential MSC markers, m-progenitors isolated from groups M, O, and M+O (day 10) were similar in expression of CD73. m-Progenitors in the group M+O expressed a slightly higher percentage of CD105 than the other two groups. In contrast, the expression of CD90 by m-progenitors in groups O and M+O was less than half of that in group M (Fig. 2).
The expression \( CD56 \), representing myogenic progenitors, in group \( M \) was significantly greater than in groups \( O \) and \( M+O \) \( (p < .001) \). PDGFR-\( \alpha \), a specific marker of M-MSCs, was expressed by \( m \)-progenitors in all three groups, but the expression of \( CD56 \) in group \( M \) is much greater than the other two groups, and groups \( O \) and \( M+O \) expressed more PDGFR-\( \alpha \) than group \( M \).

**Expression of Osteogenesis-Related Genes by \( m \)-Progenitors in Groups \( M \), \( O \), and \( M+O \)**

\( m \)-Progenitors isolated on day 1 after surgery, after culture in an osteogenic medium for 3 weeks, expressed similar quantities of type I collagen among groups \( M \), \( O \), and \( M+O \) \( (p > .05) \). Osteogenic \( m \)-progenitors obtained on days 3, 5, and 10 after surgery showed a trend of increasing expression of type I collagen as the muscle collection delayed, except for group \( M \) at day 10. \( m \)-Progenitors from group \( M+O \) expressed a higher level of type I collagen than the other two groups, but the differences in type I collagen expression among groups were not statistically significant.

Osteogenic \( m \)-progenitors of group \( M+O \) expressed the highest level of osteocalcin among the three groups, but this upregulation was only significant compared with groups \( M \) and \( O \), when the muscle samples were harvested on day 10 \( (p < .05) \). In group \( M+O \), the expression of osteocalcin by the \( m \)-progenitors isolated on days 5 and 10 was increased from day 1 \( (p < .05 \text{ and } .001, \text{ respectively}) \). Intra-group difference in the expression of osteocalcin among \( m \)-progenitors isolated at different time-points was not detected in both groups \( M \) and \( O \).

Expression of RUNX2 was significantly greater in group \( M+O \) \( (p < .001) \). RUNX2, a bone transcription factor, was expressed by \( m \)-progenitors in all three groups, but the expression of RUNX2 in group \( M \) was significantly greater than in groups \( O \) and \( M+O \) \( (p < .05) \). In group \( M+O \), the expression of RUNX2 by the \( m \)-progenitors isolated on days 5 and 10 was increased from day 1 \( (p < .05 \text{ and } .001, \text{ respectively}) \). Intra-group difference in the expression of RUNX2 by \( m \)-progenitors isolated at different time-points was not detected in both groups \( M \) and \( O \).

Table 1. List of primers

| Gene          | GenBank no. | Forward | Reverse                  |
|---------------|-------------|---------|--------------------------|
| Collagen 1    | M24372.1    | AGAGCACTACGAGTGCTGTG | ACCAGAAGTACCCCATCCCAT |
| Osteocalcin   | L24431.1    | GGTATGTGCTCCTCCGGGTTTC | CTCGGCCATCCTAAAAAGCAGA |
| RUNX2         | AH09404.2   | GCAAAATCAGATGGCCGTGG | TAGTTGGGAGCAGGAGGAGG |
| BMP2          | NM_007553.3 | GGGACCGCCTGTCCTTCTAGT | TCTCGCTGTCGGCAGCATAA |
| BMPR1A        | NM_009758.4 | GATGGTTCCGCGAGGTTGTA | ACGCATTAAACACCTCTGTT |
| Noggin        | NM_008711.2 | GAGGAGGGAAAGGCTGTC | CCGGATCCGAGATTACTCC |
| DAN           | D50263.1    | GCACAATGCTTGGTGCTTG | GAAACAGGGCGACGTTG |
| ALP           | NM_007431.3 | GGCCGTCCTAGTGTTGGGA | GTGGTTCACCCGAGGCTAG |
| \( \beta \)-Actin | NM_007393.5 | ATGGTGGATCAGCAAGCAGGA | AGGGTGAAACGCGAGCTCA |
| 18S           | NR_003278.3 | GGCCTGGTTAGTGTTGGG | TCAATCTCGGCTGGCTGAA |

Abbreviations: ALP, alkaline phosphatase; BMP2, bone morphogenetic protein 2; BMPR1A, bone morphogenetic protein receptor 1A; DAN, differential screening-selected gene aberrative in neuroblastoma.

Figure 1. Histology of the gluteus maximus and medius collected on postsurgery day 10. Compared to the normal muscle, the muscle sample of group \( M \) has significant degeneration, whereas the muscle of group \( O \) appears normal. In group \( M+O \), the muscle is similarly degenerated as in the group \( M \). There is a significant amount of calcium deposited in the muscle of group \( M+O \) \( (\text{von Kossa staining}) \). Scale bar = 50 \( \mu \text{m} \). Abbreviations: \( M \), group \( M \) (muscle injury); \( M+O \), group \( M+O \) (muscle injury and osteotomy); Norm, normal muscle; \( O \), group \( O \) (osteotomy).

Figure 2. The expression of cell surface markers by \( m \)-progenitors. \( m \)-Progenitors in all three groups expressed the common mesenchymal stem cell markers, but the expression of \( CD105 \) in group \( M+O \) is slightly higher than the other two groups, and \( CD90 \) is significantly higher in group \( M \) than the other two groups. \( CD56 \) and PDGFR-\( \alpha \) are expressed by \( m \)-progenitors in all three groups, but the expression of \( CD56 \) in group \( M+O \) is much greater than the other two groups, and groups \( O \) and \( M+O \) expressed more PDGFR-\( \alpha \) than group \( M \). \( *, p < .05; **, p < .001 \). Abbreviations: \( M \), group \( M \) (muscle injury); \( M+O \), group \( M+O \) (muscle injury and osteotomy); \( O \), group \( O \) (osteotomy); PDGFR-\( \alpha \), platelet-derived growth factor receptor \( \alpha \).

The expression \( CD56 \), representing myogenic progenitors, in group \( M \) was significantly greater than in groups \( O \) and \( M+O \) \( (p < .001) \). PDGFR-\( \alpha \), a specific marker of M-MSCs, was expressed by \( m \)-progenitors in all three groups, but the expression of \( CD56 \) in group \( M \) is much greater than the other two groups, and groups \( O \) and \( M+O \) expressed more PDGFR-\( \alpha \) than group \( M \).
The expression of BMP1A, BMP2, noggin, DAN, and ALP was evaluated in the osteogenic m-progenitors of groups M, O, and M+O isolated on day 10 after surgery. BMP1A expression by the osteogenic m-progenitors was increased in group O as compared with group M+O (Fig. 4). The osteogenic m-progenitors of group M expressed approximately two times as much BMP2 as that in groups O and M+O. Noggin expression by the m-progenitors in group M was two times that in groups O and M+O, but this was not statistically significant. DAN expression by osteogenic m-progenitors was increased in group O as compared with groups M and M+O. ALP expression by osteogenic m-progenitors of group M+O was more than three times that of groups M and O.

Osteogenesis of m-Progenitors in Groups M, O, and M+O

After culture in an osteogenic medium for 3 weeks, m-progenitors derived from gluteus maximus and medius in groups M, O, and M+O on postsurgery days 1, 3, 5, and 10 showed varied degrees of matrix mineralization, as indicated by Alizarin red staining (Fig. 5A). The cultures of these cells in the control (regular) medium did not stain for mineralization. The quantity of Alizarin red in the osteogenic cultures of the m-progenitors was influenced by muscle harvesting time point (p < .001, two-way ANOVA), but not grouping, i.e., M, O, or M+O conditions. Compared with the corresponding nonosteogenic controls, the osteogenic cultures of m-progenitors from groups O and O+M harvested on postsurgery day 10 had increased Alizarin red stain (Fig. 5B).

Contribution to Osteogenesis by the Subpopulations of m-Progenitors

Compared with a positive control of osteogenic MC3T3-E1 cells, RUNX2 expression by osteogenic m-progenitors of group M+O isolated on postsurgery day 10 was confirmed by flow cytometry. The number of m-progenitors that coexpressed RUNX2 and PDGFR-α was significantly greater than those coexpressed RUNX2 and CD56 (Fig. 6).

Discussion

This study followed the leads of clinical data that bone fracture and muscle injury are commonly associated with traumatic HO and simulated these conditions in mouse models: muscles were directly injured mechanically in group M, whereas bone damage/fracture was produced by osteotomy in group O. The greater trochanter was chosen for osteotomy because of its proximity to the gluteus maximus and medius that were to be sampled for m-progenitor isolation and clinical relevance [32]. The attachment of gluteus maximus and medius to the greater trochanter could be a conduit of inflammatory signals. Therefore, the gluteus maximus and medius in group O were not “normal” and could be inflamed indirectly by bone damage of the greater trochanter. In group M+O, the gluteus maximus and medius were both injured directly and influenced by bone fracture indirectly.

The tissue injury models in groups M, O, and M+O created distinct osteogenic environments in the gluteus maximus and medius, where a significant amount of calcium were deposited in the group M+O at postsurgery day 10. It has been known that BMP signaling induces ossification in the muscle [22]. In a mouse HO model, blunt injury increased the expression of BMP-2 and -4, BMP receptor 1, SOX9, and RUNX2 in the quadriceps, but those osteogenic signals were apparently insufficient to induce HO [33]. Similarly, muscle injury alone in group M did not lead to extensive calcium deposition in the gluteus maximus and medius.

The subsequent analyses of m-progenitors isolated from the gluteus maximus and medius demonstrated that muscle injury, osteotomy, and a combination of the two changed the makeup of the m-progenitors. In this study, m-progenitors were isolated from the gluteus maximus and medius by using an established protocol for isolation of myogenic progenitors and M-MSCs [28, 29]. This conventional plastic-adhesion method, however, resulted in a heterogeneous population of cells. The m-progenitors in the current study could include muscle interstitial progenitors, MSCs, pericytes, and satellite cells [34]. The m-progenitors of groups M, O, and M+O shared
common MSC markers but differed in expression of other cell surface markers. Progenitors in the muscle can be divided into two functional categories: myogenic progenitors or satellite cells and M-MSCs, which are capable of forming any type of cells in the muscle [12, 13]. CD56 has been found in largely myogenic progenitors, and PDGFR-α represents a group of more potent M-MSCs in the isolated muscle progenitors [14–17, 35, 36]. In this study, CD56-positive cells were predominant in the m-progenitors of group M, and PDGFR-α-positive cells were more populous in group M+O. This subpopulation shift in the m-progenitors could influence their differentiation, including osteogenic potential. Muscle repair/regeneration follows muscle injury [37]. Progenitors, including CD56-positive cells, are activated in traumatized muscles [35]. An increase of CD56-positive cells, which are largely myogenic progenitors [36], in the m-progenitors of group M, is expected. The mechanism behind the increase of PDGFR-α-positive M-MSCs in the m-progenitors in groups O and M+O, however, may be more complex [38].

The unique tissue environments in groups M, O, and M+O had different impacts on the osteogenic potential of m-progenitors. After osteogenic culture, m-progenitors isolated from the muscles in groups M, O, and M+O at different postsurgery time points upregulated osteogenic marker genes, such as type I collagen, osteocalcin, and RUNX2, to various degrees. However, it was only the m-progenitors of group M+O isolated on postsurgery day 10 that demonstrated an increased osteogenic potential over the control cultures. This was confirmed by the quantification of matrix mineralization. Collectively, the osteogenic potential of the m-progenitors in group M+O was enhanced when the muscles were harvested on postsurgery day 10, but not the earlier time points. This suggests that a regulatory tissue environment progressively influenced the differentiation potential of m-progenitors in the M+O model. Similarly, in fracture healing, bone formation is achieved following a tightly regulated cascade of molecular and cellular events [24].

Increased matrix mineralization was observed in the osteogenic cultures of both groups O and M+O. One in common between groups O and M+O was the osteotomy of greater trochanter. Previous studies have suggested that bone marrow MSCs or osteogenic progenitors from a fracture site may leak into the surrounding muscles to form HO [8]. It was unlikely in the current study, because the osteogenic potential of m-progenitors in groups O and M+O was not significantly increased until postsurgery day 10.

An imbalanced BMP and BMP antagonist signaling in the muscles could be decisive in HO formation [24, 39]. The expression of genes related to BMP signaling was compared among the m-progenitors of groups M, O, and M+O isolated on postsurgery day 10. The osteogenic m-progenitors of group M increased BMP2 expression. BMP antagonists, such as noggin (it was increased in group M, but not statistically), could block BMP signals. The overall osteogenic effect of BMP signals is a sum of BMP and BMP antagonists. An increased expression of DAN, which blocks BMP-2 and -4 extracellularly [25], could inhibit osteogenesis of m-progenitors in group O. It is generally believed that BMPR1A as the receptor of BMP is a critical element of the BMP signaling pathway. It was significantly upregulated in group O, but did not lead to significant osteogenesis in the same group. A relevant finding is that BMPR1A knockout has a positive impact on bone formation [40]. Among the three groups, the osteogenic m-progenitors in group M+O expressed the highest level of ALP, consistent with the increased expression of other osteogenic marker genes (type I collagen, RUNX2, and osteocalcin) and increased matrix mineralization. It is possible that an increased osteogenic potential of m-progenitors in group M+O is due to relatively reduced expression of BMP antagonists, such as DAN. A comprehensive analysis of the expression BMP and BMP antagonists is required to depict the osteogenic regulation in the gluteus maximus and medius in M+O model, where ossification has a high probability to occur.

m-Progenitors in group M+O isolated on postsurgery day 10 were the most osteogenic and featured an increased PDGFR-α-positive subpopulation and reduced CD56-positive subpopulation. The relation between myogenic progenitors and M-MSCs is a key in muscle degeneration [34] and could be a decisive factor in the formation of HO. Although CD56-positive myogenic progenitors differentiated to osteogenic lineage in vitro [41], they failed to form bone in vivo [14]. In osteogenic induction, multi-potent progenitors isolated from traumatized muscles did not reach terminal differentiation [42]. In other studies, those progenitors did not differentiate into osteogenic lineage at all, even under the influence of BMP-2 [43]. The inconsistence of myogenic progenitors in osteogenic differentiation was largely due to varied experimental settings and may also indicate that they are not strongly osteogenic. The m-progenitors in group M had a large portion of CD56-positive myogenic progenitors and were generally weak in osteogenic differentiation, as judged by the expression of osteogenic marker genes and matrix mineralization in osteogenic cultures.

PDGFR-α-positive cells are multipotent, depending on tissue environments. They participated in muscle regeneration in
a model of muscle injury, but leaned to adipogenesis in a model of muscle degeneration [16]. M-MSCs differentiate into osteogenic lineage under the influence of BMPs [43]. In osteogenic cultures, the expression of RUNX2 was colocalized with a greater portion of PDGFR-α-positive cells than CD56-positive cells. This suggests that PDGFR-α-positive cells in the m-progenitors of group M+O might be the source of increased osteogenesis.

The M+O model combined muscle injury and fracture. If a bone is surrounded by muscles, it is rare that a fracture is not accompanied by muscle injury. The injured muscle contributes to the tissue environment inducing HO formation [44]. For example, BMP-9 is a strong osteogenic inducer but only induces HO in injured muscles [45]. As injured muscles begin to repair, chemokines are accumulated locally and attract stem cells/progenitors [46, 47]. This phenomenon was observed in a closed fracture model, where a remarkable amount of circulating MSCs deposited in the muscles surrounding the fracture site [48]. It is likely that the tissue environment in group M+O not only is osteogenic, but also enhances MSC recruitment to the injured muscles. Both conditions are favorable to HO formation.

This study focused on the molecular and cellular events in the early stage of HO development, particularly the potential roles of myogenic progenitors and M-MSCs. There are many versions of cell surface marker combinations for identification/enrichment of the two populations of cells in the muscles. This study used CD56 and PDGFR-α to show cell subpopulation shifting in the isolated m-progenitors under the influence of different injury models. CD56 (also known as neural cell adhesion molecule) and PDGFR-α (a receptor of a potent growth factor) are common, but not unique, markers of myogenic progenitors or M-MSCs in the muscles. In this study, CD56 and PDGFR-α indicated the presentation of myogenic progenitors and M-MSCs in the isolated m-progenitors proportionately. It is noteworthy that the neuroregulation of HO formation [49] was not analyzed in the current study. In a BMP-induced HO model, the origin of osteoblastic claudin 5-positive cells were traced back to the endoneurium in peripheral nerves [50]. Interestingly, those cells participated in ossification also expressed PDGFR-α.
CONCLUSION

In summary, the injury models of M, O, and M+O created in mice induced a subpopulation shift in m-progenitors isolated from gluteus maximus and medius and influenced their osteogenic potential. m-Progenitors in group M+O isolated on postsurgery day 10 had enhanced osteogenesis in vitro, suggesting that a combination of bone and muscle injuries results in favorable tissue conditions for HO formation.

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