

Determination of cell fate in skeletal muscle following *BMP* gene transfer by *in vivo* electroporation

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differentiate into osteogenic cells. Commitment towards either muscle regeneration or induction of ectopic bone formation appears to occur five to seven days after *BMP* gene transfer.

Introduction

Bone morphogenetic protein (*BMP*) is known to have strong osteogenic potential when the recombinant protein or the *BMP* gene is transferred into skeletal muscle.¹ We previously developed a novel method for gene transfer, based on transcutaneous *in vivo* electroporation.² We applied this method to transfer the *BMP* gene and induce ectopic bone formation in skeletal muscle. Our gene transfer system with *in vivo* electroporation was applied transcutaneously to the calf muscles of rats, so that only the areas to which electrodes were attached were targeted for gene transfer. Electroporation was conducted at 100 V, with eight 50-ms pulses. This condition did not result in skin burns.^{2,3} In our previous studies, ectopic bone formation was seen to start ten days after *BMP* gene transfer, as evidenced by intramembranous or endochondral ossification being restricted to the targeted areas of skeletal muscle.³ Another gene transfer system expressing the *BMP-2* gene from an adenoviral vector induced the formation of ectopic bone in nearly all calf muscles.⁴ Therefore, the *BMP-2* gene transfer system with *in vivo* electroporation could induce both ectopic bone formation and muscle regeneration in calf muscles.^{2,3}

These results revealed that commitment to osteogenic differentiation was determined within ten days of *BMP* gene transfer and consequent muscle regeneration. At present, it is still unclear which cell types can differentiate into osteogenic cells and cause ectopic bone formation following *BMP* gene transfer by *in vivo* electroporation in skeletal muscle.

Skeletal muscle has high potential for self-regeneration. If the muscle is damaged following mechanical injury or toxic insult, satellite cells step in to repair damaged muscle fibres.⁵ Two types of stem cells with the potential to differentiate into osteogenic cells are present in skeletal muscle.⁶⁻¹⁰ On one hand, skeletal muscles harbour several muscle-derived stem-like satellite cells, which can differentiate into muscle fibres, osteogenic cells, and adipocytes.⁶⁻⁹ On the other hand, bone marrow-derived peripheral blood cells are recruited to skeletal muscles via blood flow, thereby differentiating into muscle fibres and osteogenic cells.¹⁰

In this study, we focused on the muscle-

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derived stem cell population responsible for directing muscle regeneration or bone induction after *BMP* gene transfer by *in vivo* electroporation. We tried to determine how and when these cells are committed to osteogenic differentiation as opposed to muscle regeneration by comparing them to skeletal muscle electroporated with an empty plasmid.

Abstract

We previously developed a novel method for gene transfer, which combined a non-viral gene expression vector with transcutaneous *in vivo* electroporation. We applied this method to transfer the bone morphogenetic protein (*BMP*) gene and induce ectopic bone formation in rat skeletal muscles. At present, it remains unclear which types of cells can differentiate into osteogenic cells after *BMP* gene transfer by *in vivo* electroporation. Two types of stem cells in skeletal muscle can differentiate into osteogenic cells: muscle-derived stem cells, and bone marrow-derived stem cells in the blood. In the present study, we transferred the *BMP* gene into rat skeletal muscles. We then stained tissues for several muscle-derived stem cell markers (*e.g.*, Pax7, M-cadherin), muscle regeneration-related markers (*e.g.*, Myod1, myogenin), and an inflammatory cell marker (CD68) to follow cell differentiation over time. Our results indicate that, in the absence of *BMP*, the cell population undergoes muscle regeneration, whereas in its presence, it can

Materials and Methods

BMP-2 gene transfer to skeletal muscles of rats using *in vivo* electroporation

Nine-week-old male Wistar rats ($n = 3$ per group) were purchased from Shimizu Experimental Animal (Kyoto, Japan) and maintained under specific pathogen-free conditions in our animal facility. Rats were anesthetised by intraperitoneal injection of sodium pentobarbital (5.0 mg per 100 g of body weight). The fur on the target area of the leg was removed with clippers. We injected the *BMP-2* gene expression vector, pCAGGS-*BMP-2* (0.5 $\mu\text{g}/\mu\text{L}$), at the centre of the calf muscle and immediately electroporated at 100 V with eight 50-ms pulses

(Gene Pulser®, Ohta Inc., Okayama, Japan). The control group was injected with the empty vector, pCAGGS (0.5 µg/µL), under the same conditions as above. All rat procedures were performed in accordance with Okayama University guidelines (OKU2015-137).

Immunohistochemistry

We observed time course sections one, three, five, and seven days after electroporation with the human *BMP-2* gene or empty vector. We then stained the sections with haematoxylin-eosin and several muscle-derived stem cell markers, such as paired box 7 (Pax7) and M-cadherin; muscle regeneration markers, such as myogenic differentiation 1 (Myod1) and myogenin; an inflammatory cell marker (CD68); and human *BMP-2*. Anti-Pax7 rabbit polyclonal, anti-Myod1 mouse monoclonal, anti-myogenin mouse monoclonal, anti-M-cadherin goat polyclonal, and anti-BMP-2 mouse monoclonal antibodies (all 1:100; Abcam, Cambridge, UK), and anti-CD68 mouse monoclonal antibody (1:50; Bio-Rad, Hercules, CA, USA) were incubated for 12 h at 4°C. Sections were then rinsed several times and incubated with a peroxidase-conjugated secondary antibody (1:200; Sigma, St. Louis, MO, USA) for 1 h at room temperature. Finally, sections were washed with phosphate-buffered saline, immersed in 0.05 M Tris-HCl buffer (pH 7.6, 100 mL) supplemented with 3,3'-diaminobenzidine tetrahydrochloride (20 mg) and 30% H₂O₂, and incubated for 10 min at room temperature.

Results

Day one after gene transfer

We observed several haematoxylin-positive cells (Figure 1a, black arrows) as well as damaged or lost muscle fibres (Figure 1a, red arrows) in sections of skeletal muscles transferred with the empty vector. Many CD68-positive cells had appeared in the spaces between muscle fibres (Figure 1b, blue arrows) or had migrated to some of them (Figure 1b, red arrows). M-cadherin could be detected in cells located around damaged muscle fibres (Figure 1c, arrows). Sections of skeletal muscles transferred with the *BMP-2* gene, were also rich in haematoxylin-positive (Figure 1 d,e) and *BMP-2*-positive cells (Figure 1f, red arrows). CD68-positive cells exhibited a similar localisation as in muscles transferred with the empty vector (Figure 1g,

arrows). Pax7, a marker of satellite stem cells, was detected in some muscle fibres (Figure 1h, arrows).

Day three after gene transfer

The spaces between muscle fibres appeared enlarged and filled with lymphocyte-like cells showing condensed nuclei (Figure 2 a,c, arrows) following transfer with both empty vector and *BMP-2* gene. CD68-positive cells could also be observed following both transfer procedures (Figure 2 b,d). *BMP-2*-positive cells were found only in the less damaged muscle fibres and were absent from strongly damaged ones (Figure 2e, arrows). Myod1, one of the most

important factors in early muscle regeneration, was observed in cells surrounding the spaces between muscle fibres after *BMP-2* gene transfer (Figure 2f, arrows).

Day five after gene transfer

Following transfer with the empty vector, we found a spindle-shaped and cytoplasm-enriched cell population in the spaces between muscle fibres (Figure 3a, arrows), as well as a limited number of CD68-positive cells (Figure 3b, arrow), suggesting reduced migration of inflammatory cells. Cells in the area surrounding the spindle-shaped cells, were positive for Pax7 (Figure 3c, arrow) and Myod1 (Figure 3d,

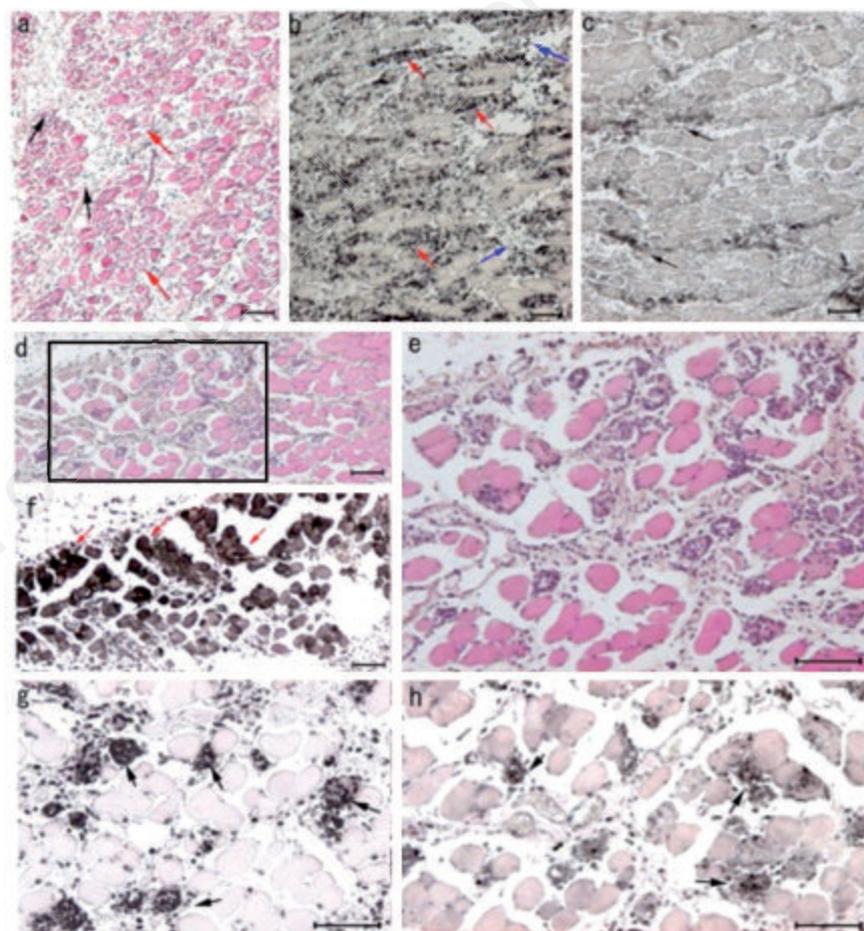


Figure 1. Histological changes in skeletal muscles on day one after empty vector or *BMP-2* gene transfer. a-c) Skeletal muscles on day one after empty vector transfer. a) Haematoxylin-eosin staining showing migration of inflammatory cells (black arrows) and muscle damage (red arrows). b) CD68-positive cells (red arrows) are detected in the spaces between muscle fibres (blue arrows). c) M-cadherin-positive cells (arrows). d-h) Skeletal muscle one day after *BMP-2* gene transfer. d) Haematoxylin-eosin staining. e) Magnification of the insert in (d). f) Human *BMP-2*-positive cells (red arrows). g) CD68-positive cells (arrows). h) Pax7-positive cells (arrows). Scale bars: 100 µm.

arrow). Myogenin, a late-stage marker of skeletal muscle regeneration, was detected in cells surrounding the spaces between muscle fibres (Figure 3e, arrows).

In contrast, skeletal muscles transferred with the *BMP-2* gene presented a canonical elongated muscle fibre appearance, and included haematoxylin-positive cells in the patterned interspaces between muscle lining (Figure 3f). Compared with that at three days after gene transfer, we found fewer CD68-positive cells (Figure 3g). Interestingly, haematoxylin-stained and lymphoid-like cells found in the spaces between muscle fibres were also BMP-2-positive (Figure 3h), even though their appearance differed from that on day three. Furthermore, Pax7 (Figure 3i, arrows), M-cadherin (Figure 3j, arrows), and myogenin (Figure 3k, arrows) could be detected around these cells.

Day seven after gene transfer

In muscles transferred with the empty vector (haematoxylin-eosin staining in Figure 4a), no CD68-positive cells could be detected (Figure 4b), whereas Pax7-positive cells localised around normal muscle fibres (Figure 4c, arrow). In skeletal muscles transferred with the *BMP-2* gene, cell migration appeared similar to that observed five days after transfer with the empty vector. The cells were spindle-shaped and cytoplasm-enriched (Figure 4d, arrows). Additionally, we could detect a few CD68-positive cells in the small spaces between muscle fibres (Figure 4e, arrows). Pax7-positive cells were observed in the spindle-shaped cell migration area, which also presented abundant extracellular matrix and muscle fibres (Figure 4f, arrows), as well as some alkaline phosphatase-positive cells (Figure 4g, arrows).

Discussion

In this study, we tried to elucidate what types of cells were recruited and how they migrated when osteogenesis and muscle regeneration occurred simultaneously following *in vivo* gene transfer with an empty or a *BMP-2*-expressing vector. To achieve this, we detected cell migration by performing a time course immunohistochemistry analysis of muscle-derived stem cells, inflammation-related cells, and muscle regenerative factors. Muscle-derived satellite stem cells play a crucial role in muscle regeneration,^{11,12} interacting with fibroblasts¹³ and inflammatory cells.¹⁴⁻¹⁸ We observed previously that, following vector injection by *in vivo* electroporation, skeletal

muscles exhibited inflammatory reaction and muscle damage.^{2,3,19,20} The inflammatory reaction peaked three days after gene transfer, but terminated by the seventh day. These inflammatory reactions were observed in skeletal muscles not only following transfer with the empty plasmid but also with the *BMP-2* gene. In the latter, ectopic bone formation was induced on the tenth day after gene transfer, whereby cartilaginous and intramembranous bone tissues were identified within the fibrous tissue that had formed between muscle fibres.³ Based on these previous data, we hypothesised that commitment to either osteogenic differentiation or muscle regeneration occurred within seven days after gene transfer, at which point the inflammatory reaction was over. To better understand the timing of the

commitment to either fate, we used several histological markers: Pax7, a marker of satellite cells in skeletal muscles;²¹⁻²³ M-cadherin, a marker of mesenchymal stem cells in skeletal muscles;^{21,24} Myod1, a marker of early-stage muscle regeneration;²¹⁻²⁴ and myogenin, a marker of late-stage muscle regeneration^{21,24} plus CD68 as a marker of inflammatory cells.^{21,25}

For up to three days, skeletal muscles transferred with either the empty vector or the *BMP-2* gene presented similar histological profiles, characterised by an ongoing inflammatory reaction. At the same time, M-cadherin-positive mesenchymal stem cells and Pax7-positive satellite cells were seen to migrate to the inflamed areas. Moreover, on the third day, muscle regeneration appeared to be already under way as

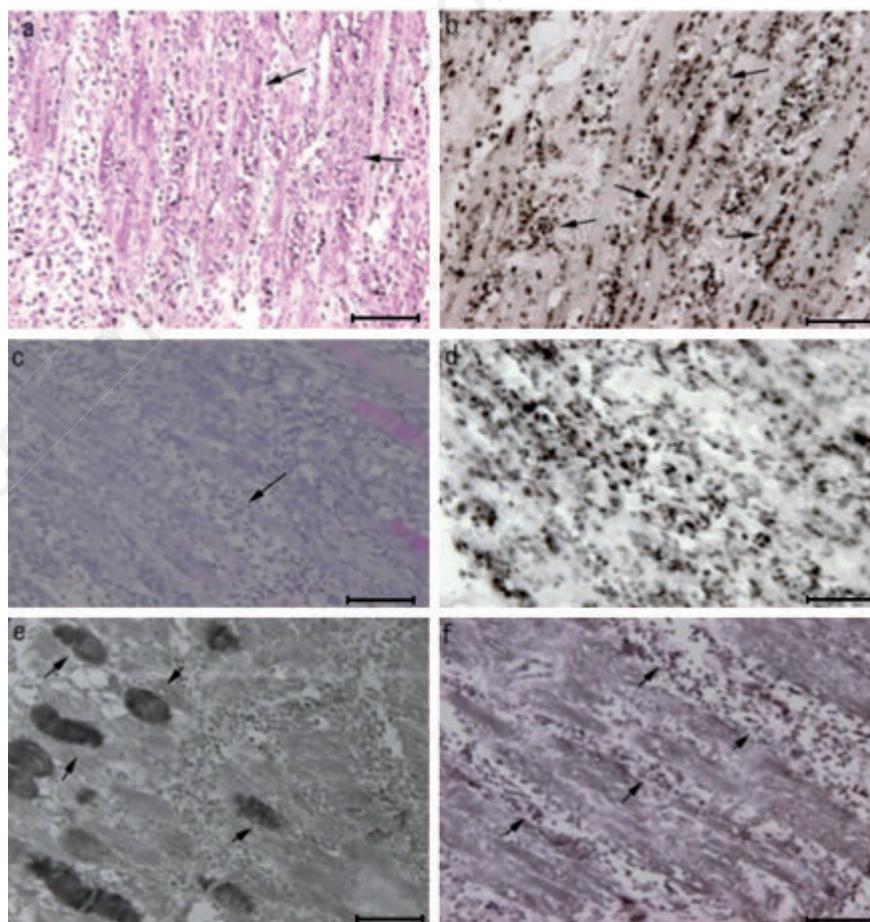


Figure 2. Histological changes in skeletal muscles on day three after empty vector or *BMP-2* gene transfer. a,b) Skeletal muscles on day three after empty vector transfer. a) Haematoxylin-eosin staining showing enlarged muscle fibres filled with lymphocyte-like cells (arrows). b) CD68-positive cells (arrows). c-f) Histological changes on day three after *BMP-2* gene transfer. c) Haematoxylin-eosin staining showing enlarged spaces between fibres (arrow). d) CD68-positive cells. e) *BMP-2*-positive cells are detected only in less damaged muscles (arrows). f) Myod1-positive cells localised around the spaces between muscle fibres (arrows). Scale bars: 100 μ m.

evidenced by Myod1-positive cells in the inflamed areas. On the fifth day, a population of spindle shaped and cytoplasm-enriched cells was observed in the spaces between skeletal muscles. These cells were Pax7- or Myod1-positive, indicating their involvement in skeletal muscle regeneration. We did not find these cells in the inter-spaces of skeletal muscles transferred with the *BMP-2* gene; however, we could still detect some CD68-positive cells and several haematoxylin-stained lymphoblast-like cells. Moreover, these lymphoblast-like cells were also BMP-2 positive. For up to three days, BMP-2 positive cells were barely detected in less damaged muscle fibres. On the fifth day after gene transfer, muscles treated with the empty vector were in the middle of the regeneration process. Instead, muscles treated with the *BMP-2* gene had started to differentiate and triggered the migration of BMP-2-positive cells to the spaces between muscles fibres. On the seventh day, skeletal muscle regeneration was almost complete in muscles transferred with the empty vector. In contrast, in skeletal muscles transferred with the *BMP-2* gene, we found CD68- and Pax7-positive cell populations, as well as alkaline phosphatase-positive cells. This indicated that these cell populations changed their direction of differentiation, favouring osteogenesis over muscle regeneration.

At first, gene transfer by *in vivo* electroporation induced an inflammatory reaction, whereby skeletal muscle-derived satellite stem cells migrated and contributed to muscle regeneration. Skeletal muscle regeneration was complete within seven days. In spite of eliciting an inflammatory reaction, *BMP-2* gene transfer by *in vivo* electroporation did not result in sufficiently severe damage within the first three days. Thus, on the fifth day, lymphoblast-like BMP-2-positive cells, rather than skeletal muscle cells, could be detected and seen to populate the spaces between muscle fibre linings. At the same time, skeletal muscle regeneration was also seen to progress simultaneously in some skeletal muscle fibres. On the seventh day, a spindle-shaped and cytoplasm-enriched cell population was observed in the interspace of skeletal muscle fibres, together with extracellular matrix containing fibrous tissue. Moreover, alkaline phosphatase-positive cells and satellite cells could be detected in these skeletal muscles. Therefore, induction of ectopic bone formation in skeletal muscles using a *BMP-2* gene expression vector and *in vivo* electroporation depends on the sequential appearance of inflammatory cells, myogenic stem cells, BMP-2-

positive cells, and finally osteogenic cells.

Taken together, our results suggest that the period between the fifth to the seventh day after *BMP-2* gene transfer by *in vivo* electroporation represents the turning point determining commitment to osteogenesis in

skeletal muscles. Moreover, Pax7-positive muscle-derived satellite cells appear to play an important role not only during muscle regeneration but also during osteogenesis in the presence of exogenous *BMP-2*. Future studies using the present gene transfer sys-

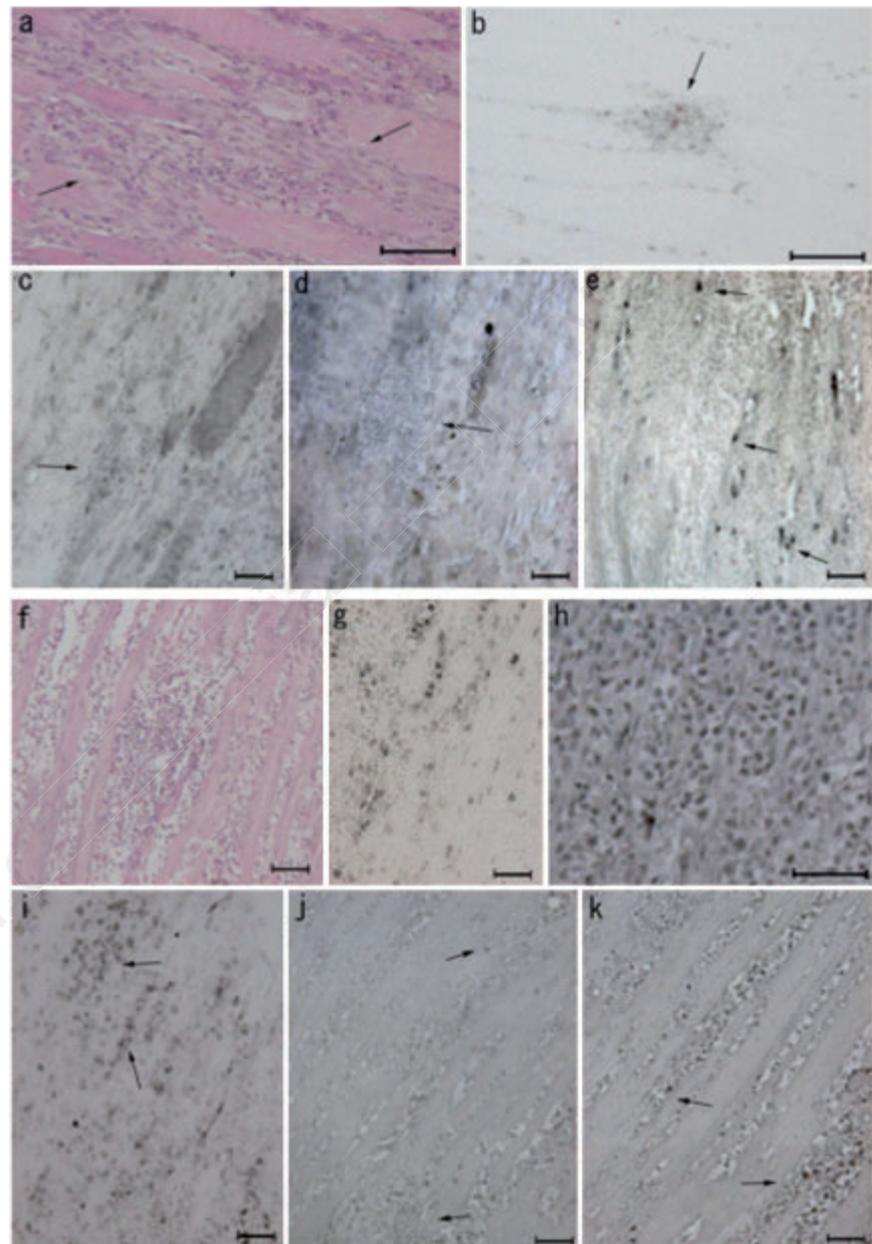


Figure 3. Histological changes on day five after empty vector or *BMP-2* gene transfer. a-e) Skeletal muscles on day five after empty vector transfer. a) Haematoxylin-eosin staining showing spindle-shaped and cytoplasm-enriched cells in the spaces between muscle fibres (arrows). b) CD68-positive cells (arrow). c) Pax7-positive cells (arrow). d) Myod1-positive cells (arrow). e) Myogenin-positive cells are detected around the cell migration area (arrows). f-k) Skeletal muscles on day five after *BMP-2* gene transfer. f) Haematoxylin-eosin staining showing typical muscle fibres. g) CD68-positive cells. h) *BMP-2*-positive cells are detected in the haematoxylin-positive population, but not in muscle fibres. i) Pax7-positive cells (arrow). j) M-cadherin-positive cells (arrows). k) Myogenin-positive cells (arrows) are detected between muscle fibres. Scale bars: a,b) 100 μm ; c-k) 50 μm .

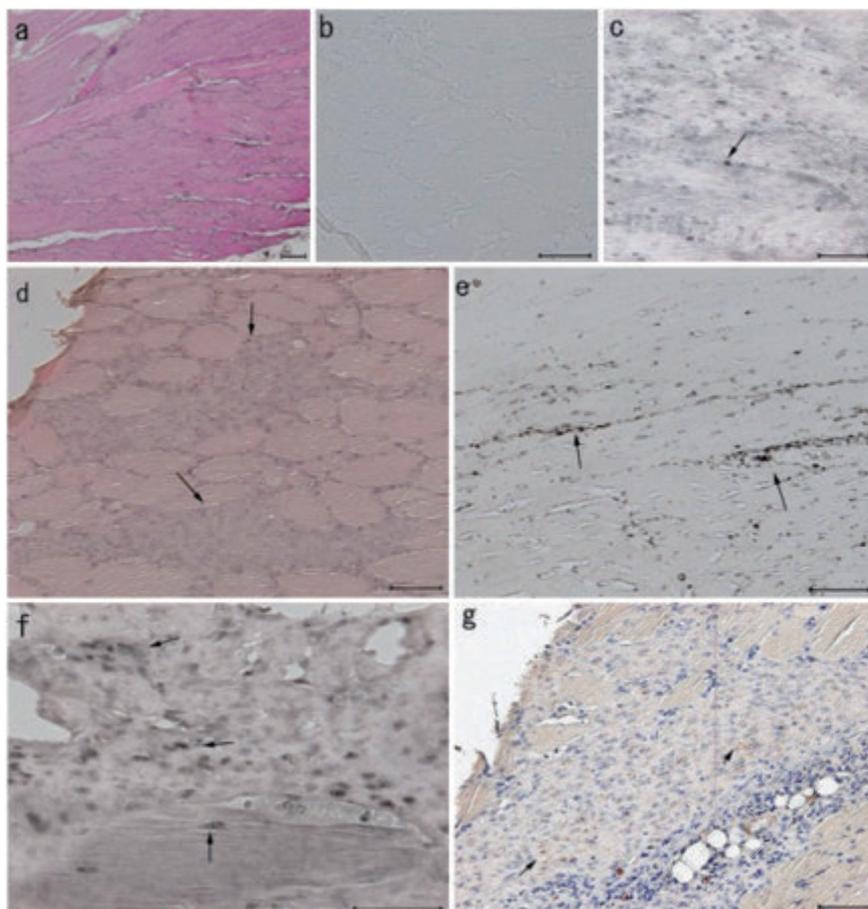


Figure 4. Histological changes on day seven after empty vector or *BMP-2* gene transfer. a-c) Skeletal muscles on day seven after empty vector transfer. a) Haematoxylin-eosin staining. b) No CD68-positive cells are detected. c) Pax7-positive cells localised around normal muscle fibres (arrow). d-g) Skeletal muscles on day seven after *BMP-2* gene transfer. d) Haematoxylin-eosin staining showing migrating spindle-shaped cells (arrows). e) CD68-positive cells (arrows). f) Pax7-positive cells localised to the spindle-shaped cells migration area (arrows). g) Alkaline phosphatase-positive cells localised to the spindle-shaped cell population (arrows). Scale bars: 100 µm.

tem, should focus on controlling the order defining cell recruitment, migration, and differentiation, as well as the production and control of a meaningful mass of bone for each individual patient.

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