Stem Cell Research & Therapeutics
SCRT, 1(1): 6-15
www.scitcentral.com

Original Research: Open Access

Osteogenic Differentiation of Skeletal Muscle-Derived Multipotent Stem Cells In A Murine Model of Tibial Bone Fracture

Yoshiyasu Uchiyama[1,2], Tetsuro Tamaki [2*], Maki Masuda[1,2] and Joji Mochida[1]

[1] *Department of Orthopedic Surgery, Tokai University School of Medicine, Japan*

[*2] *Muscle Physiology and Cell Biology Unit, Department of Human Structure and Function, Japan.*

Received November 4 2015; Accepted December 17, 2015; Published July 26, 2016

ABSTRACT

The osteogenic differentiation potential of mouse skeletal muscle-derived stem cells, sorted as $CD34^+/45^-$ (Sk-34) and $CD34^-/45^-$ (Sk-DN), was examined by direct transplantation in an experimental tibial bone fracture model. Freshly isolated Sk-34 and Sk-DN cells cultured for 5 days were obtained from GFP transgenic-mice using mild enzymatic treatment, without any induction of osteogenic differentiation. Cell pellets were directly implanted into the fractured tibial bone model generated by drilling-removal of 1/2 the circumference of the bone matrix. Concurrently, transplantations of muscle-derived $CD45^+$ cells (residual population after sorting of Sk-34 and Sk-DN cells), bone-marrow stromal cells (BMSCs), and medium without any cells, were also performed as control experiments. After 2 months, favorable bone healing was achieved in all 5 groups, suggesting the possibility of natural healing in the present model. However, active engraftment of GFP^+ cells was observed in Sk-34 (9/9) and Sk-DN (6/9) group by macroscopic fluorescence stereomicroscopy, but no GFP^+ cells were detected in the other 2 groups. Immunohistochemical analysis showed frequent presence of GFP^+/osteocalcin$^+$ cells (putative osteoblasts) in the bone matrix of the Sk-34 group. The same trend was also observed in the Sk-DN and BMSC group, but the detectable number of these cells was relatively lower than that observed with Sk-34. At 6 months after transplantation, GFP^+ donor cells with a line-cell like structure, located on the inner surface of the bone matrix were still observed in the Sk-34 group. A similar trend was also observed in both the Sk-DN and BMSC groups, but GFP^+ cells were fewer in number in these groups. These results indicate that Sk-34 and Sk-DN cells can differentiate into osteoblasts in vivo following bone fracture, in a manner similar to BMSCs. They exert their original "milieu-dependent differentiation capacity" without being inducted to the osteogenic lineage ex vivo, and are committed to the bone re-modeling cycle even after 6 months of transplantation.

Keywords: Bone fracture, Stem cell therapy, Regenerative medicine, Osteocalcin.

Abbreviations: MDSCs: Skeletal Muscle-Derived Stem Cells**,** Sk-SCs: Skeletal Muscle-Derived Multipotent Stem Cells**,** Sk-34: $CD45^-/34^+$**,** Sk-DN: $CD45^-/34^-$**,** GFP: Green Fluorescent Protein**,** DMEM: Dulbecco's Modified Eagle's Medium**,** FCS: Fetal Calf Serum**,** bFGF: Basic Fibroblast Growth Factor**,** PCR: Polymerase Chain Reaction
PBS: Phosphate Buffered Saline, PFA: Paraformaldehyde, PB: Phosphate Buffer, EDTA: Ethylene Diamine Tetra Acetic Acid, ALP: Alkaline Phosphatase, BMSCs: Bone-Marrow Stromal Cells, DAPI: 4,6-Diamino-2-Phenylindole, BMP: Bone Morphogenetic Protein, FBS: Fetal Bovine Serum.

INTRODUCTION

The bone-autograft is the most common method to heal large-size bone defects in orthopedic therapy, whereas an additional invasive surgical procedure may lead to donor site morbidity. Therefore, it is difficult to obtain a sufficient volume of bone autograft for a critically large-sized defect. In this case, although allografts have been considered, there are concerns about disease transmission and immune rejection. To overcome this problem, a large number of synthetic grafts such as metallic implants, ceramics, polymers, and composites of these materials have been developed [1,2]. However, the problem with these is the absence of osteogenicity (supply of bone-forming cells), osteoinductivity (initiation of cell differentiation), and osteocondutivity (facilitation of cell and nutrient infiltration).

Corresponding author: Tetsuro Tamaki, Ph.D, Muscle Physiology and Cell Biology Unit, Department of Human structure and Function, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan, Tel: +81-463-93-1121 (ext.2524); Fax: +81+463-95-0961; E-mail: tamaki@is.icc.u-tokai.ac.jp

Citation: Uchiyama Y, Tamaki T, Masuda M, Mochida J. (2016) Osteogenic Differentiation of Skeletal Muscle-Derived Multipotent Stem Cells In A Murine Model of Tibial Bone Fracture. Stem Cell Res Th, 1(1): 6-15.

Consequently, the recent trend is focused on calcium phosphate-containing synthetic biomaterials such as hydroxyapatite relating substances which show properties of osteoinductivity [3], but the results of their sole use are still not satisfactory. For this purpose, fabricated synthetic biomaterials or scaffolds co-transplanted with osteogenic stem cells, were considered to be capable grafts with all three biological capacities mentioned above.

Several stem cells including bone marrow stromal cells [4], adipose tissue-derived stem cells [1], and skeletal muscle-derived stem cells (MDSCs) have been used for healing bone defects. However, from the clinical point of view, heterotopic ossification in the skeletal muscle has been well-known as an example of ectopic bone formation in the soft-tissue [5,6] . In this context, stem, and/or progenitor cells from the skeletal muscle are likely to have somewhat of an advantage than the others. In fact, accelerated bone formation after MDSC transplantations has been reported [7,8], whereas the level of their commitment to bone formation, such as a detailed analysis of the fate and differentiation of the engrafted cells is still not clear.

We also fractionated mouse skeletal muscle-derived multipotent stem cells (Sk-SCs), as CD45-/34$^+$ (Sk-34) and CD45$^-$/34$^-$ (Sk-DN or Sk-DN/29$^+$) cells, using original and mild enzymatic isolation and fluorescence activated cell sorting (FACS) [9,10]. These cells were capable of synchronized reconstitution of the muscle-nerve-blood vessel unit associated with the capacity to differentiate into skeletal muscle cells, Schwann cells, perineurial/endoneurial cells, pericytes, vascular smooth muscle, and endothelial cells after in vivo transplantation into various tissues [11-16]. We also fractionated the human skeletal muscle-derived Sk-34 and Sk-DN/29$^+$ cells using the same method as above, and demonstrated that they have comparable differentiation and tissue reconstitution capacities to mouse cells [17]. However, the osteogenic capacity of Sk-34 and Sk-DN cells is still unknown as was observed in MDSCs derived from the pre-plating culture system derived [7,8].

Therefore, the purpose of this study is to clarify whether mouse Sk-34 and Sk-DN cells can differentiate into osteogenic cells, and facilitate bone formation after in vivo transplantation. For this purpose, we developed the bone defect model in the tibia, and demonstrated that both Sk-34 and Sk-DN cells upon transplanting, differentiated into osteoblastic cells and remained committed to the bone metabolic cycle for over 6 months.

Materials and Methods

Animals

Green fluorescent protein transgenic mice (GFP-Tg mice; C57BL/6 TgN[act EGFP]Osb Y01, provided by Dr. M. Okabe, Osaka University, Osaka, Japan) [18] were used as donor mice (male, 4-8 week-old, n=12), and wild-type mice (C57BL/6N) were used as recipients (male, 8-12 week-old, n=29) in the transplantation experiments. All experimental procedures were approved by the Tokai University School of Medicine Committee on Animal Care and Use.

Purification and preparation of transplanted Cells

Muscle sampling was performed under an overdose of pentobarbital (60 mg/kg, Schering-Plough, + butorphanol tartrate 2 mg/kg, Meiji Seika, Tokyo, Japan, i.p.). The thigh and lower leg muscles (tibialis anterior, extensor digitorum longus, soleus, plantaris, gastrocnemius, and quadriceps femoris) of GFP-Tg mice were removed and used for subsequent experiments. The average total muscle mass removed was 509 ± 47 mg/GFP-Tg mouse (mean ± SE). Sk-SCs were isolated according to the previously described procedure [9,10]. Briefly, muscles were not minced, and were then treated with 0.1% collagenase type IA (Sigma-Aldrich, Tokyo, Japan) in Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) containing 7.5% fetal calf serum (FCS, Equitech Bio, TX, USA) with gentle agitation for 2 h at 37°C. After digestion, isolated cells were serially filtered through 70-, 40-, and 20-μm nylon filters, in that order, to remove debris. Cells were stained against CD45 and CD34, and sorted as Sk-34 (CD45$^-$/34$^+$), Sk-DN (CD45$^-$/34$^-$) cells and CD45$^+$ cells. Sk-34 and CD45$^+$ cells were freshly prepared for the transplantation experiment. However, Sk-DN cells were expanded in a collagen-based culture medium (CollagenCult H4742, StemCell Tech., Vancouver, Canada) with 10 ng/ml bFGF and 20 ng/ml EGF for 5 days before the transplantation, because of their small number and immaturity [10,19,20].

In addition, whole bone-marrow stromal cells (BMSCs) were obtained by flushing the tibias and femurs of GFP mice (n=2-3/experiment). After elimination of red blood cells, mononuclear bone marrow cells were obtained, and cultured in 20%FCS/DMEM for 48 hours. Floating cells were eliminated and the remaining adhesive cells were cultured further for 5 days (total of 7 days). Media were changed after every 2 days for both cultures. Expanded BMSCs were transplanted in the same manner as both Sk-SCs.

Furthermore, CD45$^+$ cells, which were sorted at the same time for other two Sk-SCs and were considered as contaminated hematopoietic cells from circulating blood, were also transplanted as a control group. Transplantation of the same amount of media without cells was also performed to evaluate the present bone fracture model. Consequently, we transplanted 5 types of cells: (1) freshly isolated Sk-34 cells (n=9), (2) freshly isolated CD45$^+$ cells (n=4), (3) expanded Sk-DN cells (n=9), (4) expanded BMSCs (n=4), and (5) medium without cells (n=3).

RT-PCR for Sk-MSCs before and after transplantation

In order to test the expression of specific markers of cell differentiation and paracrine capacities as the putative indicators of osteogenic cells, bulk cell RT-PCR was performed. Specific primers and the materials analyzed are summarized in **Table 1**. Just prior to transplantation, some cells were lysed and total RNA was purified using a QIAGEN RNeasy micro kit. First-strand cDNA synthesis was performed with an Invitrogen SuperScript III system using dT30-containing primers (see above), and specific PCR (35 cycles of 30 seconds at 94°C, 30 seconds at 60-65°C and 2 minutes at 72°C) was performed in a 15-µl reaction containing ExTaq buffer, 0.8 U of ExTaq-HS-polymerase, 0.7 µM specific sense and antisense primers, 0.2 mM dNTPs, and 0.5 µl cDNA. β-actin was used as the house-keeping control.

Table 1. Specific primers for RT-PCR.

Gene Name	Product size	Forward Primer	Reverse Primer
Runx2	294	GAGGCCGCCGCACGACAACCG	CTCCGGCCCACAAATCTCAGA
Osteorix	183	GGTCCAGGCAACACACCTAC	GGTAGGGAGCTGGGTTAAGG
Type I collagen	76	CTGGCTTTGCCGGCC	ACCTTTAACACCAGTATCACCAGGT
ALP2	66	TCAGGGCAATGAGGTCACATC	CACAATGCCCACGGACTTC
Osteocalcin	275	CTCTCTCTGCTCACTCTGCT	AATAGTGATACCGTAGATGCGTT
Osteoprotegerin	577	TCCTGGCACCTACCTAAAACAGCA	CTACACTCTCGGCATTCACTTTGG
Osteopontin	190	CATCCCTGTTGCCCAGCTTC	TTCCAGGCTGGCTTTGGAAC
β-actin(Beta actin)	93	TCAGCAAGCAGGAGTACGATGAG	AAAGGGTGTAAAACGCAGCTCAG

Preparation of the bone defect model on the tibia

In order to generate the experimental bone defect, the left tibia of the mice was exposed after skin incision and blunt dissection of the periosteum and muscles. This was followed by an intramedullary nailing from the proximal end using a 27G syringe needle. After retraction of the skin and the muscles, a square-hole (2 x 2mm) was bored with an electric drill occupying almost 1/2 of the whole circumference (**Figure 1B**). The hole was then filled with the medium containing each above cell type (3-4µl with 1x 10^5 cells/µl, **Figure 1A**). The transplanted portion was covered by replacing the periosteum and muscles, and suturing the skin.

Macroscopic Observation and Immunostaining

At 2 and 6 months after transplantation, recipient mice were given an overdose of pentobarbital (60 mg/kg, i.p) + xylazine HCl (10 mg/kg, i.p.), and the engraftment of donor-derived GFP^+ cells in the damaged portion of the tibial bone was confirmed by fluorescence stereomicroscopy (SZX12; Olympus, Tokyo, Japan) (see **Figure 3A**). Recipient mice were then perfused with warm 0.01 M phosphate buffered saline (PBS) through the left ventricle, followed by fixation with 4% paraformaldehyde/0.1 M phosphate buffer (4% PFA/PB). The tibial bone was then removed and fixed overnight in 4% PFA/PB at 4°C followed by washing with a graded sucrose (0–25%)/0.01 M PBS series continuously containing 0.25M EDTA during the course of 1 week. Samples were then immersed in OCT compound and frozen/stored at -80°C. Subsequently, 7-µm histological sections were prepared. Localization of osteoblasts was detected by goat polyclonal anti-osteocalcin (dilution = 1:1800; incubation=4°C overnight; Biomedical technologies, Stoughton, MA), and alkaline phosphatase staining (ALP staining kit, Mutoh chemical, Tokyo, Japan). Reactions were visualized using Alexa Fluor-594-conjugated rabbit anti-goat antibodies (1:500, room temperature for 2 h; Molecular Probes, Eugene, OR). Nuclei were counter-stained with DAPI (4,6-diamino-2-phenylindole).

RESULTS

Evaluation of the experimental bone fracture model

Favorable healing of bone fracture was observed in the medium control group, and so this model can be considered as a naturally healing model. In this context, this experiment is a confirmation study of how transplanted cells may contribute and/or commit to bone formation following a natural healing process.

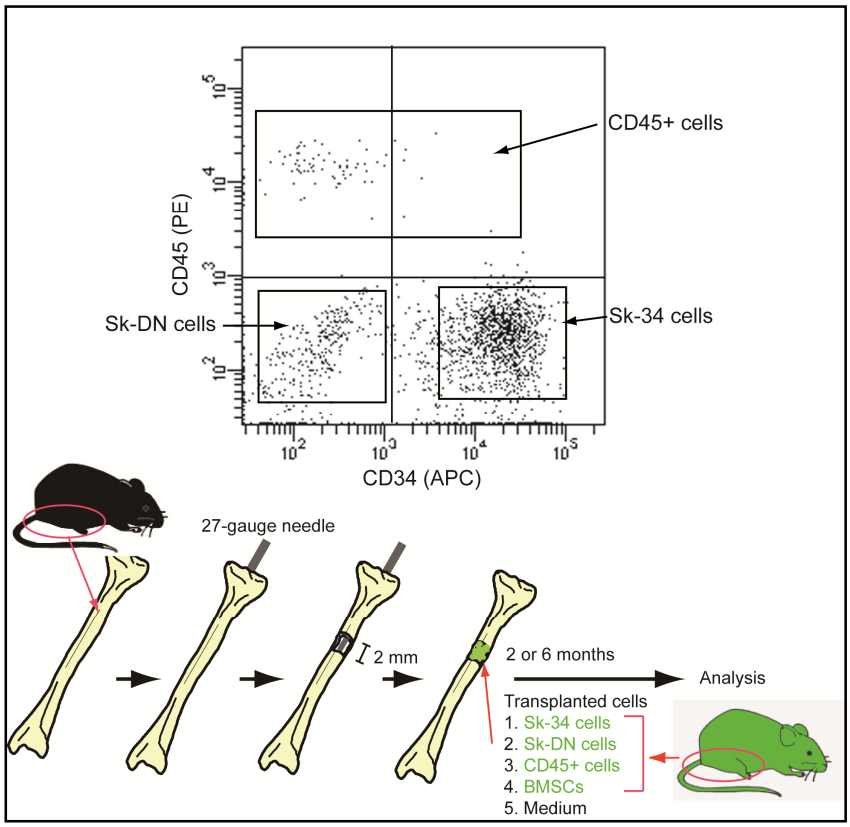

Figure 1. Sk-SC sorting, generation of the bone fracture model, and cell transplantation. (A) Typical sorting of Sk-34, Sk-DN, and CD45$^+$ cells from GFP-Tg mice. (B) Schematic depicting the generation of a bone fracture model in the tibia followed by transplantation of sorted GFP$^+$ cells. Sk-34 - skeletal muscle-derived CD34$^+$/45$^-$ cells; Sk-DN - CD34$^-$/45$^-$ cells; BMSCs - bone marrow-derived stromal cells.

Putative osteogenic capacity of Sk-34, Sk-DN cells, and BMSCs just before transplantation

The putative osteogenic capacity of Sk-34, Sk-DN, and BMSCs just before transplantation was determined by RT-PCR analysis (**Figure 2**). The mRNA expression of seven markers specific for osteogenic cells was examined in freshly isolated Sk-34 cells, Sk-DN cells cultured for 5 days, and BMSCs expanded for 7 days. Results indicate that all three cells showed almost equal expression of all markers except for alkaline phosphatase (lane No. 4), which was lacking in Sk-DN cells. Therefore, all three cells may show putative osteogenic capacities before transplantation that seems relatively higher in Sk-34 cells and BMSCs.

Macroscopic fluorescence stereomicroscopy

Macroscopic fluorescence stereomicroscopy showed no GFP$^+$ emissions in the BMSCs and CD45$^+$ transplanted bones, even though bone healing was complete. However, in the Sk-34 and Sk-DN cell-transplanted groups, clear GFP emissions were consistently observed. Typical engraftment of GFP$^+$ tissues in the tibial bone at 2 months after Sk-34 and Sk-DN cell transplantations is shown in **Figure 3A and 3B**. The damaged portion showed a thicker circumference than the normal portion, and GFP$^+$ cells were strictly incorporated in the bone tissues of the recipient (arrows). Detection of GFP$^+$ tissue-engraftment was observed in 9/9 instances of Sk-34 and in 6/9 instances of Sk-DN transplantation. In addition, the Sk-DN transplantation group a showed relatively smaller volume of GFP$^+$ tissues compared to the Sk-34 group.

Histological analysis

Histological analysis of the damaged tibial portion in Sk-34 cell transplantation is shown in **Figure 3C** and **D**. Immunohistochemical detection using anti-osteocalcin clearly indicated that several GFP$^+$ cells in the bone matrix close to the marrow are osteocalcin$^+$ (arrows in **Fig. 3C**). These are considered as early stage osteocytes, suggesting that the transplanted donor Sk-34 cells could differentiate into osteoblasts and contribute to bone matrix formation. Similarly, several GFP$^+$ cells also showed alkaline phosphatase positive reactions in the marrow area, thus providing further evidence of osteoblast differentiation (arrows in **Figure 3D**).

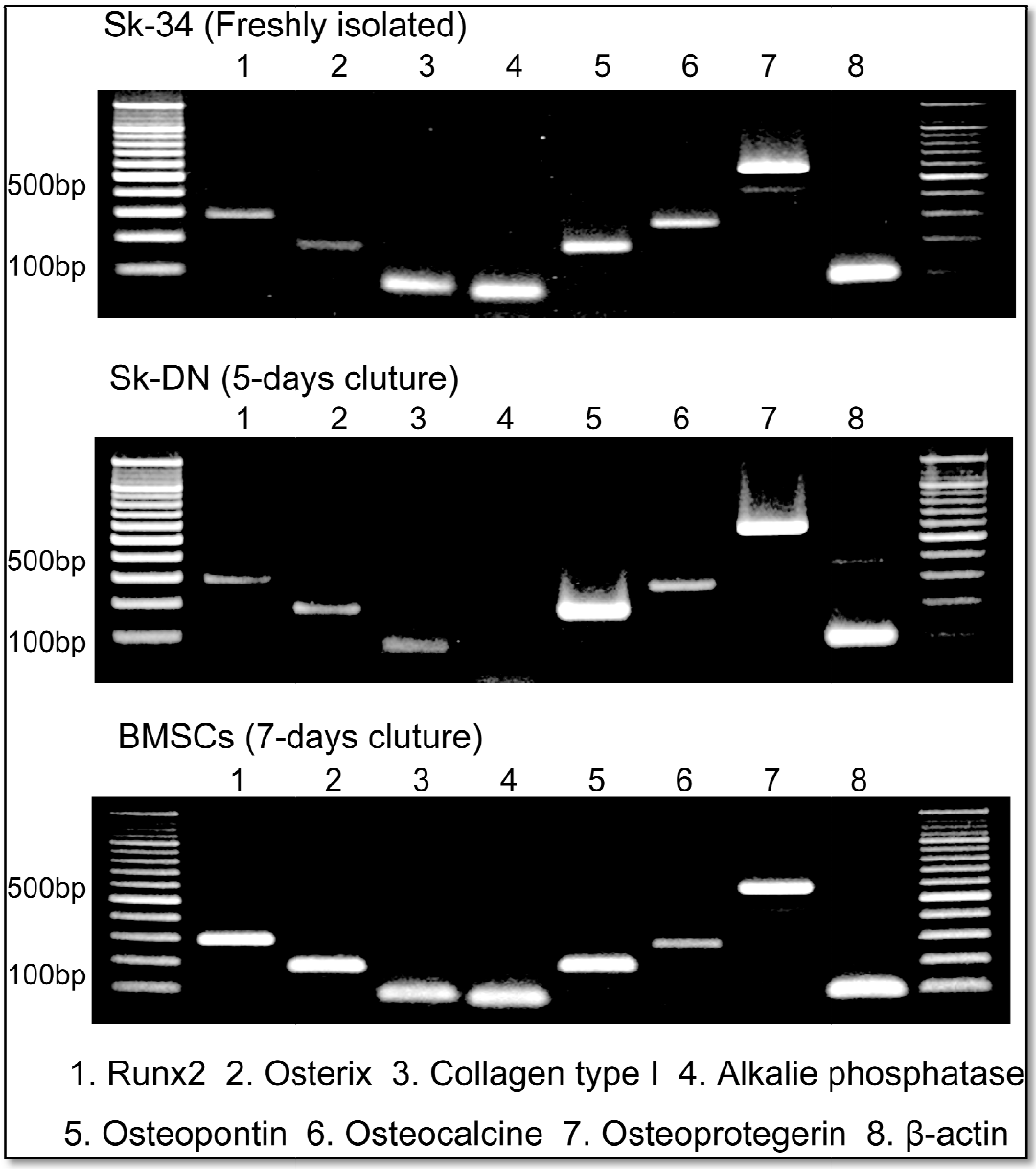

Figure 2. RT-PCR of freshly isolated Sk-34 cells, Sk-DN cells expanded for 5 days, and BMSCs expanded for 7 days just before transplantation. The putative osteogenic capacity is same in all the cell types. Bp = base pair.

Similar patterns were also observed in the Sk-DN cell and BMSCs transplantation group, but the number of cells involved in these was relatively fewer than Sk-34, as revealed by the macroscopic observations above.

Furthermore, GFP^+/$osteocalcin^+$ cells were detected even at 6 months after transplantation. Donor-derived $osteocalcin^+$ and GFP^+ cells lined up on the border between the bone matrix and marrow (**Figure 4A-C**), thus considered as pre-osteoblasts. A similar trend but with few cells was observed in the Sk-DN group. This result suggests that donor-derived GFP^+ cells can continuously reside for 6 months in vivo, and participate in the bone metabolic cycle or in the bone re-modeling process. In fact, comparable behavior such as formation of GFP^+ bone lining-cell like structures (negative for osteocalcin), was also observed in BMSC transplantation at 6 months (**Figure 4D-F**), although the corresponding detectable area was smaller than in Sk-34 cell transplantation.

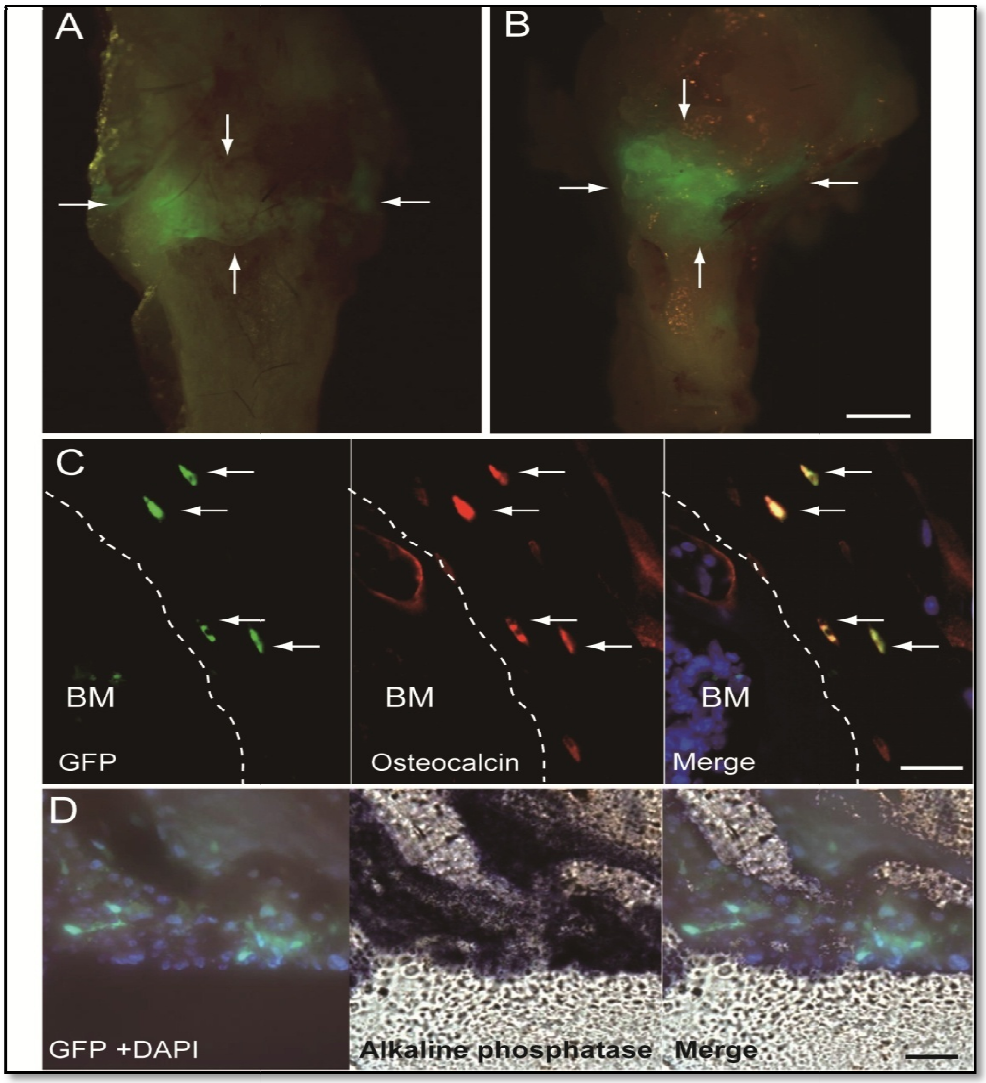

Figure 3. Histological analysis of Sk-34 cell-transplanted portion of the tibia at 2 months after the operation. Macroscopic fluorescence stereomicroscopy for Sk-34 (A) and Sk-DN (B) cell-transplanted portions. (C) Immunohistochemical detection of GFP^+/$osteocalcin^+$ cells in Sk-34 cell-transplanted bone matrix (arrows). Dotted line shows the border between the bone matrix and marrow. (D) Alkaline phosphatase staining of the Sk-34 cell-transplanted portion. Location of GFP^+ cells corresponds to dark staining of alkaline phosphatase. Blue staining in panel C and D - DAPI, BM - bone marrow, Bars in A and B = 1mm and those in C and D = 20 µm.

DISCUSSION

The present study clearly demonstrated that Sk-SCs, which were sorted as Sk-34 and Sk-DN cells, differentiated into osteoblasts after direct and separate transplantation into fractured bone. In addition, the present result also demonstrated that engrafted Sk-34 cells aligned serially on the inner surface of the bone matrix (bone formation area) were evident even after 6 months of transplantation (**Figure 4A-C**). This indicates that the engrafted donor cells thrive in the bone formation area of the cavity in a manner similar to typical pre-osteoblasts derived from the marrow stroma [21], and a similar trend was also confirmed by BMSCs transplantation in the present study (**Figure 4D-F**). In case the transplanted cells were to show only a single episode of bone formation during the early-stage of regeneration, they would be eliminated over 6 months. However, the engrafted cells were retained in the bone and showed a preserved pre-osteogenic aligning structure. This suggested the commitment of transplanted cells into the ossification cycle during 6 months.

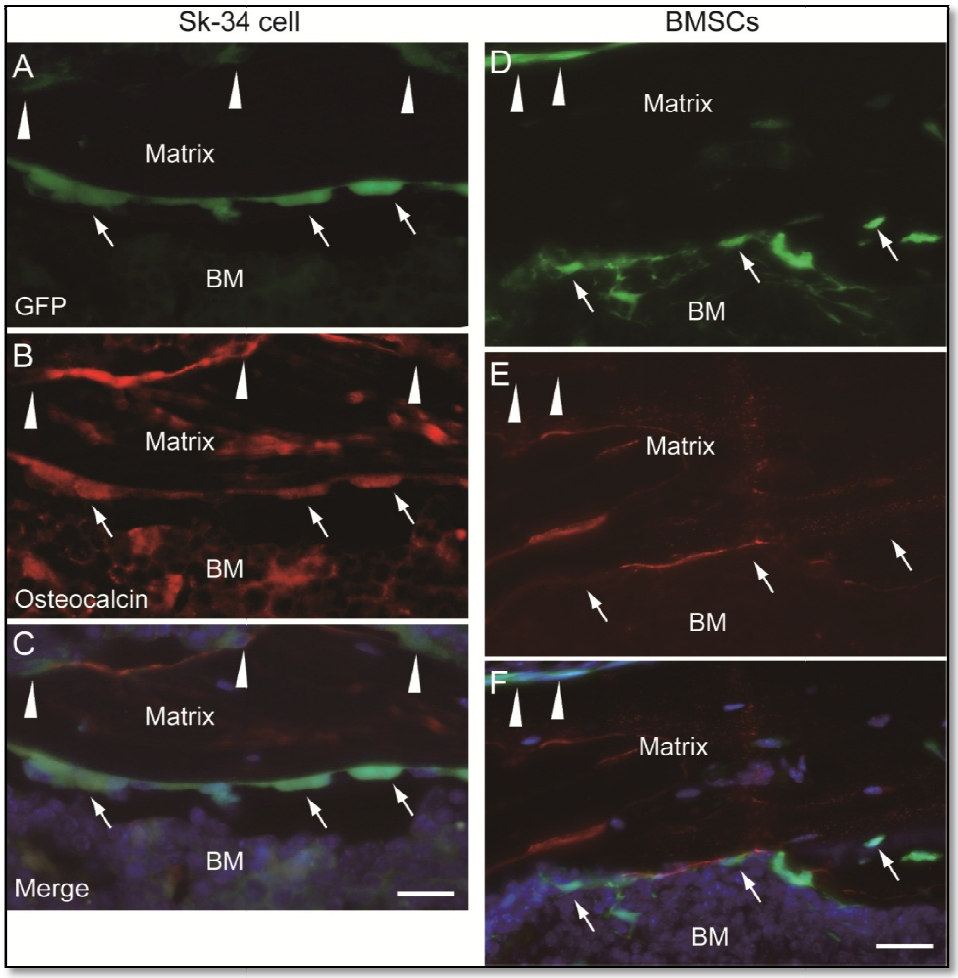

Figure 4. Immunohistochemical detection of GFP^+/$osteocalcin^+$ cells at 6 months after transplantation of Sk-34 cells and BMSCs. (A-C) Sk-34 transplantation. GFP^+/$osteocalcin^+$ cells aligned in the area of bone formation (inside surface of matrix; arrows in A-C). GFP^+ cells are also observed on the outer surface of the tibia (arrowheads). (D-F) BMSCs transplantation. Similar locations of engrafted BMSCs are observed inside (arrows) and outside (arrowheads) the matrix. BM - bone marrow. Bars = 20 µm.

The applications of skeletal muscle-derived stem cells in the bone healing process have been previously reported [7,22]. However, these studies used ex vivo activation for the cells, with agents such as bone morphogenetic protein (BMP) -2, -4 -6 and -7 [23-27]. Similar methods were also applied to other stem cells, such as fibroblasts [28], bone marrow derived cells [29,30], and fat derived cells [31,32]. However, the most appropriate cell type for bone healing is still unknown. In this study, we did not subject the Sk-34 and Sk-DN cells to any ex vivo induction toward the osteogenic lineage, but the expression of mRNAs specific to the bone lineage was naturally observed in these cells before transplantation (**Figure 2**). In addition, osteogenic differentiation was not detected in the expansion cell culture system (data not shown). This indicates that the actual osteogenic differentiation was mainly induced after in vivo transplantation due to "milieu dependent differentiation". The microenvironment of fractured bone naturally comprises various ossification related factors (cytokines, chemokines, growth factors and hormones), which may naturally induce osteogenic differentiation of the transplanted Sk-SCs. To our knowledge, this is the first report of such "milieu dependent osteogenic differentiation" and "the commitment to the ossification cycle" in Sk-SCs. Therefore, we hypothesize that osteogenic differentiation potential is higher in Sk-34 and Sk-DN cells than other cells used in the present study.

With respect to the differences between the Sk-SCs used in the present study and other skeletal muscle-derived stem cells, particular consideration was given to the cell isolation method. During enzymatic digestion, proteolytic contamination can damage cell-surface ligands or receptors, which are necessary for stem cell function after in vivo transplantation [33]. Therefore, we have consistently used lower concentrations of collagenase (0.1%) in DMEM

followed by addition of 5-10% FBS to the collagenase solution in order to minimize contaminating protease activity and to protect the isolated cells. This is a mild treatment when compared with those used in previous studies, which used higher collagenase concentrations (0.2-2.0%) without suppression of protease activity, followed by dispase (0.25%) and/or trypsin (0.1-0.25%) treatments. Furthermore, in contrast to other reports, intact donor muscle was used for enzymatic digestion instead of minced tissue. These factors mostly affected in vivo differentiation potential. In addition, we recently isolated human Sk-SCs using the same method and found that they had a similar differentiation capacity as their mouse counterparts [17]. However, human Sk-SCs can be divided two particular cell populations; the Sk-DN fraction showed limited inclusion of skeletal-myogenic cells, whereas, the remaining multipotent stem cells were contained in the Sk-34 fraction [17]. Therefore, which cells can be differentiate into osteogenic cells is still unknown but interesting.

Concerning the lower engraftment ratio in Sk-DN cells, mouse Sk-DN cells are placed upstream to Sk-34 cells in stem cell hierarchy [20], and exert the same differentiation and tissue reconstitution capacities in damaged muscle-nerve-blood vessel units [15,19]. However, due to the lesser number of freshly isolated Sk-DN cells, culture expansion was necessary in this experiment. This expansion process may induce relatively higher skeletal-myogenesis in Sk-DN cells than in Sk-34, and may result in lowering the osteogenic potential of Sk-DN cells. This suggests that the process of skeletal-myogenesis is reciprocal to that of osteogenesis. In other words, inhibition of skeletal-myogenesis may induce osteogenesis in Sk-SCs.

However, critically large-sized bone defects, the combined use of scaffolds may be absolutely necessary. Therefore, materials showing affinity for cell adherence are considered to be the best sources of scaffolds. In this context, hydroxyapatite and β-tricalcium phosphate may be good candidate for treating bone fractures [1,3,34,35]. We are currently investigating the combined use of hydroxyapatite and Sk-SCs for critically large bone fractures and their capacity for bone formation.

CONCLUSION

The present data indicates that mouse Sk-SCs, sorted as Sk-34 and Sk-DN cells, differentiated into osteoblasts in the in vivo bone fracture niche after 2 months of transplantation. These cells were retained even after 6 months of transplantation and participated in the bone re-modeling cycle, as efficiently as transplanted BMSCs.

REFERENCES

1. Fellah BH, Gauthier O, Weiss P, Chappard D, Layrolle P (2008) Osteogenicity of biphasic calcium phosphate ceramics and bone autograft in a goat model. Biomaterials 29: 1177-1188.
2. Gentile P, Mattioli-Belmonte M, Chiono V, Ferretti C, Baino F, et al. (2012) Bioactive glass/polymer composite scaffolds mimicking bone tissue. J Biomed Mater Res Part A 100: 2654-2667.
3. Guda T, Walker JA, Pollot BE, Appleford MR, Oh S, et al. (2011) In vivo performance of bilayer hydroxyapatite scaffolds for bone tissue regeneration in the rabbit radius. J Mater Sci Mater Med 22: 647-656.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, et al. (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147.
5. Kocyigit H, Hizli N, Memis A, Sabah D, Memis A (2001) A severely disabling disorder: fibrodysplasia ossificans progressiva. Clin Rheumatol 20: 273-275.
6. Mahboubi S, Glaser DL, Shore EM, Kaplan FS (2001) Fibrodysplasia ossificans progressiva. Pediatr Radiol 31: 307-314.
7. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, et al. (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157: 851-864.
8. Wernig A, Zweyer M, Irintchev A (2000) Function of skeletal muscle tissue formed after myoblast transplantation into irradiated mouse muscles. J Physiol 522: 333-345.
9. Tamaki T, Akatsuka A, Ando K, Nakamura Y, Matsuzawa H, et al. (2002) Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J Cell Biol 157: 571-577.
10. Tamaki T, Akatsuka A, Okada Y, Matsuzaki Y, Okano H, et al. (2003) Growth and differentiation potential of main- and side-population cells derived from murine skeletal muscle. Exp Cell Res 291: 83-90.
11. Nitta M, Tamaki T, Tono K, Okada Y, Masuda M, et al. (2010) Reconstitution of experimental neurogenic bladder dysfunction using skeletal muscle-derived multipotent stem cells. Transplantation 89:1043-1049.
12. Hoshi A, Tamaki T, Tono K, Okada Y, Akatsuka A, et al. (2008) Reconstruction of Radical Prostatectomy-Induced Urethral Damage Using Skeletal Muscle-Derived Multipotent Stem Cells. Transplantation 85: 1617-1624.
13. Soeda S, Tamaki T, Hashimoto H, Saito K, Sakai A, et al. (2013) Functional Nerve-Vascular

Reconstitution of the Bladder-Wall; Application of Patch Transplantation of Skeletal Muscle-Derived Multipotent Stem Cell Sheet-Pellets. J Stem Cell Res Ther 3:142.

14. Saito K, Tamaki T, Hirata M, Hashimoto H, Nakazato K, et al. (2015) Reconstruction of Multiple Facial Nerve Branches Using Skeletal Muscle-Derived Multipotent Stem Cell Sheet-Pellet Transplantation. PLoS One 10: e0138371.
15. Tamaki T, Okada Y, Uchiyama Y, Tono K, Masuda M, et al. (2007) Synchronized reconstitution of muscle fibers, peripheral nerves and blood vessels by murine skeletal muscle-derived CD34(-)/45 (-) cells. Histochem Cell Biol 128: 349-360.
16. Tamaki T, Uchiyama Y, Okada Y, Ishikawa T, Sato M, et al. (2005) Functional recovery of damaged skeletal muscle through synchronized vasculogenesis, myogenesis, and neurogenesis by muscle-derived stem cells. Circulation 112: 2857-2866.
17. Tamaki T, Uchiyama Y, Hirata M, Hashimoto H, Nakajima N, et al. (2015) Therapeutic isolation and expansion of human skeletal muscle-derived stem cells for the use of muscle-nerve-blood vessel reconstitution. Front Physiol 6: 165.
18. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407: 313-319.
19. Tamaki T, Okada Y, Uchiyama Y, Tono K, Masuda M, et al. (2007) Clonal multipotency of skeletal muscle-derived stem cells between mesodermal and ectodermal lineage. Stem Cells 25: 2283-2290.
20. Tamaki T, Okada Y, Uchiyama Y, Tono K, Masuda M, et al. (2008) Skeletal muscle-derived CD34+/45- and CD34-/45- stem cells are situated hierarchically upstream of Pax7+ cells. Stem Cells Dev 17: 653-667.
21. Clarke B (2008) Normal bone anatomy and physiology. Clin J Am Soc Nephrol;3 Suppl 3: S131-S139.
22. Lee JY, Qu-Petersen Z, Cao B, Kimura S, Jankowski R, et al. (2000) Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol 150: 1085-1100.
23. Betz VM, Betz OB, Rosin T, Keller A, Thirion C, et al. (2015) The effect of BMP-7 gene activated muscle tissue implants on the repair of large segmental bone defects. Injury.
24. Wright V, Peng H, Usas A, Young B, Gearhart B, et al. (2002) BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. Mol Ther 6: 169-178.
25. Shen HC, Peng H, Usas A, Gearhart B, Cummins J, et al. (2004) Ex vivo gene therapy-induced endochondral bone formation: comparison of muscle-derived stem cells and different subpopulations of primary muscle-derived cells. Bone 34: 982-992.
26. Betz OB, Betz VM, Schroder C, Penzkofer R, Gottlinger M, et al. (2013) Repair of large segmental bone defects: BMP-2 gene activated muscle grafts vs. autologous bone grafting. BMC Biotechnol 13: 65.
27. Komaki M, Asakura A, Rudnicki MA, Sodek J, Cheifetz S. (2004) MyoD enhances BMP7-induced osteogenic differentiation of myogenic cell cultures. J Cell Sci 117: 1457-1468.
28. Krebsbach PH, Gu K, Franceschi RT, Rutherford RB (2000) Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. Hum Gene Ther 11: 1201-1210.
29. Lieberman JR, Le LQ, Wu L, Finerman GA, Berk A, et al. (1998) Regional gene therapy with a BMP-2-producing murine stromal cell line induces heterotopic and orthotopic bone formation in rodents. J Orthop Res 16:330-339. .
30. Zachos T, Diggs A, Weisbrode S, Bartlett J, Bertone A (2007) Mesenchymal stem cell-mediated gene delivery of bone morphogenetic protein-2 in an articular fracture model. Mol Ther 15: 1543-1550.
31. Guilak F, Estes BT, Diekman BO, Moutos FT, Gimble JM. (2010) 2010 Nicolas Andry Award: Multipotent adult stem cells from adipose tissue for musculoskeletal tissue engineering. Clin Orthop Relat Res 468: 2530-2540.
32. Mehlhorn AT, Niemeyer P, Kaschte K, Muller L, Finkenzeller G, et al. (2007) Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. Cell Prolif 40: 809-823.
33. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, et al. (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122:289-301.
34. Zhu W, Wang D, Xiong J, Liu J, You W, et al. (2015). Study on clinical application of nano-

hydroxyapatite bone in bone defect repair. Artif Cells Nanomed Biotechnol 43: 361-365.

35. Guo X, Zheng Q, Kulbatski I, Yuan Q, Yang S, et al. (2006) Bone regeneration with active angiogenesis by basic fibroblast growth factor gene transfected mesenchymal stem cells seeded on porous beta-TCP ceramic scaffolds. Biomed Mater 1: 93-99.