**ABSTRACT**

Bone marrow-derived mesenchymal stem cells (BMMSCs) are multipotent stem cells capable of differentiation into a variety of cell types, proliferation, and production of clinically useful secretory factors. These advantages make BMMSCs highly useful for cell transplantation therapy. However, the molecular network underlying BMMSC proliferation remains poorly understood. Here, we showed that TGFβ-activated kinase 1 (Tak1) is a critical molecule that regulates the activation of cell cycling and that Tak1 inhibition leads to quiescence in BMMSCs both in vivo and in vitro. Mechanistically, Tak1 was phosphorylated by growth factor stimulations, allowing it to bind and stabilize Yap1/Taz, which could then be localized to the nucleus. We also demonstrated that the quiescence induction by inhibiting Tak1 increased oxidized stress tolerance and improved BMMSC engraftment in intramuscular and intrabone marrow cell transplantation models. This study reveals a novel pathway controlling BMMSC proliferation and suggests a useful method to improve the therapeutic effect of BMMSC transplantation.

**SIGNIFICANCE STATEMENT**

This study demonstrates that TGFβ-activated kinase 1 (Tak1) is a critical regulator of bone marrow-derived mesenchymal stem cell (BMMSC) proliferation. Tak1 was activated by various mitogenic cytokines and could then induce stabilization and nuclear localization of Yap1/Taz by interacting with these proteins. Tak1 inhibition brought about the quiescent state in BMMSCs both in vivo and in vitro. Reversible quiescence induced by Tak1 inhibition provided stress tolerance and improved engraftment for BMMSCs in cell transplantation models.

**INTRODUCTION**

Bone marrow-derived mesenchymal stem cells (BMMSCs) hold great potential for regenerative medicine because of their capacity for self-renewal and differentiation into multiple types of cells such as adipocytes, osteocytes, and chondrocytes. To date, substantial evidence for the clinical advantages of BMMSCs have been reported. For example, it is known that these cells produce various secretory factors that support tissue repair and normalization of local inflammation [1, 2]. However, fundamental biological information regarding BMMSCs, including how they maintain their undifferentiated status and switch fate between self-renewal and differentiation, remains poorly understood. In particular, the molecular mechanisms underlying the regulation of BMMSC proliferation are unknown. The elucidation of these mechanisms is essential for controlling the condition and quality of stem cells for therapeutic uses.

According to previous studies, the regulation of proliferation of mesenchymal stem cells (MSCs) is associated with the following signaling cascades: fibroblast growth factor (FGF)- and subsequent MAP kinases (MAPK)/ERK activity-dependent signaling, platelet-derived growth factor (PDGF)/PDGF receptor-activated signaling through ERK and AKT activation [3], hepatocyte growth factor- and epidermal growth factor (EGF)-activated signaling through ERK and PI3K-AKT [4], inflammatory cytokine-activated signaling through stress-associated kinases JNK and p38, and transforming growth factor β (TGFβ) signaling via TGFβ receptor/ALK-Smads and MAPK and PI3K [5]. However, the critical molecules that regulate cell proliferation of BMMSCs have not been identified to date.

TGFβ-activated kinase 1 (Tak1), a member of the mitogen-activated protein kinase–kinase–kinase (MAP3K) family, regulates various important cellular events such as differentiation, cell death, and proliferation of cancer cells and immune cells [6–10]. In recent decades, important
roles of Tak1 in tissue development have been suggested, such as in cartilage [11, 12] and palate [13], and in self-renewal of muscle satellite cells [14–16]. It has also been demonstrated that Tak1 activity is involved in inhibition of differentiation in mesenchymal progenitors [15]. These findings evoke the idea that Tak1 function is closely associated with maintenance and/or self-renewal of stem cells in mesenchymal lineages.

In the current study, we investigated if Tak1 is important in the regulation of BMMSC self-renewal process. The results from this study are expected to provide insights for improving the therapeutic application of BMMSCs in transplantation.

Materials and Methods

Ethics Statement

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Kindai University and were performed in accordance with institutional guidelines and regulations. Human BMMSCs were provided by the RIKEN Bio-Resource Research Center following approval from the ethics committee of Kindai University (approved no. 24-089).

Isolation and Culture of BMMSCs

Six-week-old C57BL/6N male mice were used for the experiment. Bone marrow (BM) tissues were prepared as previously reported [17]. To isolate the resident BMMSCs from BM tissues, cleaned long bones were cut into small pieces around 2–3 mm³ and treated with collagenase type I (Wako, Tokyo, Japan) for 15 minutes. Then, dissociated tissues were washed twice with phosphate-buffered saline (PBS) [–] and reacted with anti-PDGF-Rα (17-1401-81, eBioscience, San Diego, CA), anti-Sca1 (61-5981-82, Thermo Fisher Scientific, Waltham, MA), anti-CD45 (35-0451-U500, TONBO bioscience, San Diego, CA), and anti-Ter119 (35-5921-U500, TONBO bioscience) for 1 hour at 4°C followed by sorted with FACS Aria II (BD Biosciences, Franklin Lakes, NJ). For culture, single cell suspensions containing BMMSCs were plated onto cell culture dishes (Sumilon, Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and cultured in α-MEM (Wako) supplemented with 200 μM l-glutamine, 10% fetal bovine serum (HyClone, Logan, UT) under 5% CO₂ and 5% O₂ at 37°C. On day 2 of culture, the medium was replaced to remove dead cells and debris. After 10 days of culture, BMMSCs that formed colonies of fibroblastic cells were disaggregated by treatment with TrypLE Express (Thermo Fisher Scientific) and evaluated by FACS using Image J software and scored for the nuclear localization index (NLI) as follows: fluorescent intensity of each protein in the DAPI+ area was measured. Then the average of fluorescent intensity of all cells in the control (avg. of control) was obtained. Each score for the test groups was divided by the average of control to obtain NLI for each protein.

Immunoprecipitation

Plasmid coding HA-tagged Tak1 (Addgene #44160), Yes-associated protein 1 (Yap1), or transcriptional coactivator with PDZ domain (Taz) were transfected into the mouse BMMSCs (mBMMSCs) using a CUY21 electroporator (NEPA Gene, Tokyo, Japan). Whole-cell lysates or fractionated samples were incubated with 2 μg of specific antibodies for 8 hours at 4°C in immunoprecipitation buffer (50 mM Tris [pH 7.3], 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100) with 1 mM PMSF and protease inhibitors (Sigma–Aldrich). Immunocomplexes were isolated with protein G-Sepharose beads saturated with 1% BSA, by gentle rocking for 4 hours at 4°C. Beads were washed five times with ice-cold Immunoprecipitation (IP) buffer. Bound proteins were retrieved from Sepharose beads by boiling in Laemmli buffer containing β-mercaptoethanol. To detect the ubiquitinated Yap1 and Taz, the cells were treated with proteasome inhibitor MG132 at 1 nM for 6 hours before sampling.

Transplantation of the BMMSCs into Muscular Tissues

The recipient B6 animals were anesthetized using 2% isoflurane. Following anesthesia, posterior biceps femoral muscles were opened aseptically, create the slit with scalpel blade. The primary BMMSCs establishes from C57BL/6-Tg (CAG–EGFP, Japan SLC, Inc., Hamamatsu, Japan) were prepared with 20 nM 5zox for 6 days, adjusted to 1.5 × 10⁶ cells per milliliter in PBS and injected 100 μl into the slit with 1 ml syringes with 27G needles. After 48 hours of the transplantation, the muscles were collected, digested, and analyzed with FACS.
Intrabone Marrow Transplantation of BMMSCs

Following anesthesia, knee joints were opened aseptically, and 27G needles were inserted into the bone cavity of tibia to create the space for cell transplantation. Primary BMMSCs collected from the EGFP male mice were pretreated with 5zox as above and adjusted to $1.5 \times 10^9$ cells per milliliter in PBS. The $5 \times 10^4$ BMMSCs were then injected into the bone cavity of tibia using a 50 μl microsyringe (Hamilton Co. Whittier, CA) as described previously [21, 22].

Statistical Analysis

Significant differences were detected by Tukey–Kramer HSD test or Student’s t test, as appropriate. $p$ values less than .05 were considered significant.

RESULTS

Inhibition of Tak1 Leads to Suppression of Proliferation of BMMSCs

To investigate the importance of Tak1 for the proliferation of BMMSCs, we treated three independent mBMMSC lines and two independent human BMMSCs (hBMMSCs and mBMMSCs, respectively) with 5zox, which is a specific inhibitor of Tak1 [23]. Upon 5zox treatment, cell proliferation and colony formation were significantly inhibited in a dose-dependent manner in both BMMSC expansion and differentiation (expansion: 5zox−, differentiation: 5zox−); these data were quantified with ImageJ and are shown as black bars in the bar graph. Middle three panels are results of differentiation induction, in which 5zox was added during BMMSC expansion, and removed during differentiation (expansion: 5zox+, differentiation: 5zox−); these data are shown as gray bars in the bar graph. Right three panels are results of differentiation induction, in which 5zox was added during BMMSC expansion and differentiation (expansion: 5zox+, differentiation: 5zox+); these data are shown as white bars in the bar graph. Asterisks represent significant differences ($p < .05$, $n = 6$) compared with the “expansion: 5zox−, differentiation: 5zox−” condition. Abbreviations: BMMSC, bone marrow-derived mesenchymal stem cells; hBMMSC, human bone marrow-derived mesenchymal stem cells; mBMMSC, mouse bone marrow-derived mesenchymal stem cells; NTC, non-treatment control.
TAK1 Is a Critical Regulator of BMMSC Proliferation

To confirm whether Tak1 inhibition suppressed cell proliferation of BMMSCs in vivo, we injected S5ox into juvenile mice. Young mice at day 10 after birth were injected with S5ox for five times every other day. When weight was measured the day after the final injection, the S5ox-treated juvenile mice (S5ox mice) showed growth retardation in weight compared with the vehicle-treated control (Fig. 2A). In the S5ox mice, the undifferentiated BMMSCs, identified as PDGFR<sup>+</sup>Sca1<sup>+</sup>/CD45<sup>−</sup>/Ter119<sup>−</sup> (PaS), were significantly decreased (Fig. 2B), indicating that inhibition of Tak1 blocked cell growth of the BMMSCs in vivo. Retarded growth observed in the S5ox mice recovered within 3 weeks of stopping Tak1 inhibition (Fig. 2C). The S5ox mice showed normal during S5ox treatment; after weight recovery and subsequent maturation, they showed normal fertility. To validate that suppression of Tak1 lead to slowing or blocking of BMMSC proliferation, we performed siRNA-mediated inhibition of Tak1 in vivo. When the cell cycle status of the PaS fraction was evaluated at 24 hours after second injection of siRNA (methods have been described in Supporting Information Materials and Methods), the population of cells in S/G<sub>2</sub>/M phases was significantly decreased, whereas the percentage of cells in G<sub>0</sub>/G<sub>1</sub> phases was significantly increased (Supporting Information Fig. S3). Based on these, we hypothesized that Tak1 is important for the cell cycle process.

Tak1 Inhibition Induces Quiescence-Like Characteristics in BMMSCs

To further elucidate the changes induced by Tak1 inhibition in BMMSCs, we examined the cell cycle status using the BMMSCs collected from Fucci<sub>1</sub>-orange/S/G<sub>2</sub>/M-green transgenic mice (Fucci-TG). Following S5ox treatment, the percentage of G<sub>2</sub>/G<sub>1</sub> cells significantly increased, and conversely, S/G<sub>2</sub>/M populations decreased (Fig. 3A and Supporting Information Fig. S4A), indicating that S5ox blocked the progression of cell cycle from G<sub>0</sub>/G<sub>1</sub> to subsequent steps. Also in vivo, treatment with S5ox brought about accumulation of G<sub>2</sub>/G<sub>1</sub> cells (Supporting Information Fig. S5). Western blot (WB) analysis for Caspase 3 showed that S5ox treatment did not induce the cleavage of Caspase 3, indicating that Tak1 may not be essential for cell viability (Supporting Information Fig. S4B). FACS-based analysis for necrosis or apoptosis using PI/Annexin V dye also demonstrated that cell death was not induced upon treatment with standard conditions of 20 nM S5ox; even after over exposure to 200 nM of S5ox, ~90% of BMMSCs survived (Supporting Information Fig. S4C).

Microarray of the Tak1-treated BMMSCs and showed that active cell cycle-associated genes encoding cyclin dependent kinases (CDKs), as well as proliferating cell nuclear antigen and mini chromosome maintenance complex component 4 (MCM4), were suppressed. In contrast, genes involved in the maintenance of quiescent status, such as those encoding Cdkn1a (p21), Cdkn1c (p57), and Cdkn2a (p16) were upregulated (Fig. 3B). Furthermore, it was found that other quiescence-associated genes, for example, antistress genes such as those encoding Sir1, Sod1, and forhead box O1, 3 (Foxo1, 3), as well as polycomb genes Bmi1, and enhancer of zeste homolog 1 (EZH1) were also upregulated by S5ox treatment by the microarray and qRT-PCR (Fig. 3C). To determine if the upregulated antioxidant genes were functional, we analyzed intracellular reactive oxygen species (ROS) with CellROX dye and found that S5ox treatment reduced the ROS levels in cultured BMMSCs after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 3D). Furthermore, subsequent cell viability assay using PI and Annexin V (AnV) dyes showed that pretreatment with S5ox reduced cell populations during early apoptosis detected as PInegative/AnVpositive from 21.1% to 9.4% and late apoptosis detected as PIpositive/AnVpositive from 4.1% to 2.1% (Fig. 3E). Together, these results suggested that pretreatment with S5ox increased the tolerance for oxidative stress in BMMSCs.

referred to as mBMMSCs hereafter. To determine the suppressive effect of Tak1 blockade against BMMSC proliferation, we performed siRNA-mediated suppression of Tak1 expression in BMMSCs. The siRNA against Tak1 (siTak1) decreased the expression level of Tak1 mRNA to ~40% of that in the untreated control (Supporting Information Fig. S2A, S2B). Approximately 60% reduction in total cell number was observed at 48 hours after siRNA transfection when compared with that of the control (Supporting Information Fig. S2C). Next, we investigated whether Tak1 inhibition alters the differentiation potential of BMMSCs. Upon S5ox treatment, both chondrogenesis and osteogenesis was inhibited, whereas no significant difference was observed in adipogenesis. When S5ox addition was discontinued before induction of differentiation, the differentiation efficiencies into chondrocytes, osteoblasts, and adipocytes were comparable with those of the untreated controls (Fig. 1C).

Tak1 Inhibition Blocks Proliferation of BMMSCs In Vivo

To further elucidate the changes induced by Tak1 inhibition in BMMSCs, we examined the cell cycle status using the BMMSCs collected from Fucci<sub>1</sub>-orange/S/G<sub>2</sub>/M-green transgenic mice (Fucci-TG). Following S5ox treatment, the percentage of G<sub>2</sub>/G<sub>1</sub> cells significantly increased, and conversely, S/G<sub>2</sub>/M populations decreased (Fig. 3A and Supporting Information Fig. S4A), indicating that S5ox blocked the progression of cell cycle from G<sub>0</sub>/G<sub>1</sub> to subsequent steps. Also in vivo, treatment with S5ox brought about accumulation of G<sub>2</sub>/G<sub>1</sub> cells (Supporting Information Fig. S5). Western blot (WB) analysis for Caspase 3 showed that S5ox treatment did not induce the cleavage of Caspase 3, indicating that Tak1 may not be essential for cell viability (Supporting Information Fig. S4B). FACS-based analysis for necrosis or apoptosis using PI/Annexin V dye also demonstrated that cell death was not induced upon treatment with standard conditions of 20 nM S5ox; even after over exposure to 200 nM of S5ox, ~90% of BMMSCs survived (Supporting Information Fig. S4C).

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Phosphorylated Tak1 Activates Cell Proliferation Through Noncanonical TGFβ Pathway Under TGFβ1 Supplementation

To elucidate the mechanisms underlying Tak1-mediated cell cycle activation in BMMSCs, we first explored molecules that could activate Tak1 in serum-dependent cultures. As candidate molecules, we choose TGFβ1, FGF, EGF, PDGF-AB, or PDGF-BB, all of which are important components found in serum for cell culture. Upon supplementation of these molecules, Tak1 was phosphorylated not only by TGFβ1, but also by FGF (bFGF), EGF, PDGF-AB, and PDGF-BB treatments (Fig. 4A). Next, we explored the downstream mediators of Tak1 using a simplified culture, in which proliferation of BMMSCs depended on only TGFβ1, and inhibitors or siRNA against candidate molecules. Under such culture condition, BMMSCs proliferated depending on the concentration of TGFβ1 (Fig. 4B). When the BMMSCs were treated with inhibitors against Tak1 (5zox), p38 (SB202190), MEK (PD0325901), and PI3K/Akt (LY-294002) in the TGFβ1-dependent culture, a decrease in cell expansion was observed. Particularly, the administration of 5zox brought about strong suppression of both colony formation (Supporting Information Fig. S6) and cell proliferation (Fig. 4C, 4D). Suppression of Smad2 and 3 did not affect BMMSC proliferation (Supporting Information Fig. S2). Erk1 and Erk2 elicited a 40% reduction in TGFβ1-induced proliferation. In contrast, suppression of Tak1 led to 70% reduction in cell proliferation (Supporting Information Fig. S2).

Figure 3. Analysis of cell cycle status in the bone marrow-derived mesenchymal stem cells (BMMSCs) treated with 5zox. (A): FACS-based quantification of cell numbers in the BMMSCs treated with vehicle (same amount of DMSO) and 5zox by the fucci G1-orange/S/G2M-green system. Asterisks represent significant differences (p < .05, n = 3) compared with control. (B): Expression change in the genes associated with cell cycle regulation found by microarray analysis. (C): qRT-PCR analysis for the expression change of the quiescence-related genes. Significant differences between nontreatment control and 5zox were detected in all genes represented here (p < .05, n = 3). (D): Reactive oxygen species detection with CellROX dye and FACS. Asterisks represent significant differences (p < .05, n = 3) compared with vehicle control. (E): Detection of apoptosis/necrosis by PI/Annexin V (AnV) staining. PI/AnV double-positive population indicates cell population undergoing necrosis or late apoptosis. PI negative/AnV positive fraction indicates cell population undergoing necrosis or early apoptosis.

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Figure 4. Effect of growth factors or inhibitors against map kinases (MAPKs) under serum-free culture conditions. (A): Phosphorylation status of TGFβ-activated kinase 1 (Tak1) under various growth factor stimulations. (B): Dose-dependent mitogenic effect of TGFβ1 on the serum-free culture of bone marrow-derived mesenchymal stem cells (BMMSCs). Cells were stained with crystal violet dye. (C): Colony formation assay after limited dilution. Cells were stained with crystal violet dye. (D): Relative cell numbers after 7 days culture under each inhibitor treatment conditions. Asterisks represent significant differences (p < .05, n = 3) compared with nontreatment control. Abbreviation: pTak1, phosphorylated Tak1.

Figure 5. Immunofluorescence for Tak1, Yap1, and Taz in the bone marrow-derived mesenchymal stem cells (BMMSCs). BMMSCs treated with DMSO or 5zox were observed using a fluorescent microscope after IF with anti-Tak1/FITC-2ndAb and anti-Yap1 or Taz/TexasRed-2ndAb. NLI indicates the nuclear localization index and the scores are shown as mean value ± SD of all cells in each image. Scale bar = 50 μm.

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Activated Form of Tak1 Localizes into the Nucleus in Proliferating BMMSCs and Binds to Yap1/Taz

To investigate the mechanism underlying Tak1-mediated BMMSC proliferation, we performed immunofluorescence (IF) analysis of Tak1. In cultured BMMSCs, TAK1 was localized in both the cytoplasm and nucleus. In contrast, when BMMSCs were treated with 5zox for 6 hours, Tak1 was excluded from the nucleus of almost all cells. NLI of each image showed that nuclear localization of Tak1, Yap1, and Taz were blocked by the 5zox treatment (Fig. 5). To confirm the change in Tak1 localization, we performed nuclear/cytoplasmic fractionation of BMMSCs followed by WB-based Tak1 detection. Tak1 was detected in both nuclear and cytoplasmic fractions from untreated samples, whereas the expression was remarkably low in the nuclear fraction from 5zox-treated BMMSCs (Fig. 6A). Since Tak1 lacks a DNA-binding domain and nuclear localization signal, we hypothesized that some interacting molecule(s) may mediate signal transduction between Tak1 activity and mitogenic reactions in BMMSCs. To identify such molecules, we focused on Yap1 and its paralog Taz, which are transcriptional activators of the Hippo tumor suppressor pathway involved in organ size regulation and tumorigenesis. To examine the possibility of interaction between Tak1 and Yap1/Taz, we performed IF and WB with anti-Tak1 and anti-Yap1 or anti-Taz antibodies. In proliferating BMMSCs, Yap1 and Taz were also expressed in both the cytoplasm and nucleus. In contrast, amounts of nuclear Yap/Taz were reduced in the 5zox-treated BMMSCs (Figs. 5 and 6A). We then examined the possibility of a direct interaction between Tak1 and Yap1/Taz using the coimmunoprecipitation (Co-IP) approach. To perform this experiment, we transfected a HA-tagged TAK1 plasmid (HA-Tak1) into BMMSCs and immunoprecipitated whole-cell lysate with anti-HA antibody. Subsequent WB assay showed that Yap1/Taz were present in the immunoprecipitated fraction of the BMMSCs (Fig. 6B). This means that both Yap1 and Taz could bind with Tak1. On the other hand, the binding between Tak1-Yap1 and Tak1-Taz were reduced by the 5zox-treatment (Fig. 6C). We hypothesized that Tak1 contributes to stabilization of Yap1/Taz by regulating its phosphorylation status. To test this, we performed WB for Yap1Ser397, which is a degradation mark of Yap1 [24] conserved in mouse cells [25]. Under normal culture conditions, Yap1Ser397 was very weakly detected. When the BMMSCs were treated with 5zox, Yap1Ser397 was strongly expressed (Fig. 6D). To confirm whether interaction with Tak1 elicited a change in the ubiquitination status of Yap1/Taz, we transfected HA-Yap1 or HA-Taz in BMMSCs and performed IP with anti-HA antibodies 48 hours after transfection with or without 5zox treatment. Under normal conditions without 5zox treatment, ubiquitinated Yap1/Taz were very weakly detected, and by 5zox treatment ubiquitinations of Yap1 and Taz were increased (Fig. 6E).

Pretreatment with 5zox Confers High Viability on Transplants by Inducing Quiescence During Transplantation of BMMSCs

We examined whether Tak1 inhibition enables efficient synchronization into the quiescent state and higher survival rate after transplantation. Prior to transplantation of the 5zox-treated BMMSCs, we determined the duration of the effect of 5zox in the BMMSCs after deprivation of 5zox from the medium. At 12 hours after 5zox removal, more than 90% of the cells were still maintained at the G0/G1 stage. At 12 hours after the second medium change, ~80% cells were retained in the G0/G1 stage. Even at 72 hours after 5zox removal, 60% of the BMMSCs were at the G0/G1 stage (Fig. 7A). Thus, we considered that 5zox treatment was effective just by treating BMMSCs before cell transplantation. To confirm that the pretreatment with 5zox improved cell survival during implantation, we injected 5zox-treated BMMSCs prepared from EGFP mice into the injured femur muscles of syngeneic wild-type B6 mice and performed PI/AnnV assays and CellROX assays for the EGFP+ cells at 48 hours after injection. FACS analysis for the EGFP+ fraction revealed that the cell population undergoing early apoptosis decreased from 82.4% to 50.1%, and the population in late apoptosis decreased from 82.4% to 8.9% (Fig. 7B). CellROX analysis showed that intracellular ROS levels were suppressed in the 5zox pretreated cells even after 48 hours of injection (Fig. 7C). Next, we examined whether the pretreatment with 5zox improved the...
long-term engraftment by performing intrabone marrow transplantation (IBMT) using the BMMSCs from EGFP mice. Approximate, 3,500 EGFP+ cells per $1 \times 10^6$ BM cells, which corresponds to 7% of the injected cells, remained at 24 hours after transplantation (Fig. 7D and Supporting Information Fig. S8). At this time point, 5zox treatment resulted in a threefold increase in the engrafted cells compared with the control. Although the numbers of engrafted cells decreased depending on days after cell transplantation, the predominancy of the engrafted rate was maintained after 5zox treatment and 2,500 cells per $1 \times 10^6$ BM cells, corresponding to 5% of the injected cells survived and engrafted at 28 days after cell transplantation (Fig. 7D). When the survived EGFP+ cells in the recipient were sorted using PDGFRα and Sca1 antibodies, we found that only 5% of the engrafted cells in the control group expressed both markers. In contrast, 40% of the engrafted cells maintained the expression of both markers in the 5zox groups (Fig. 7E).

**DISCUSSION**

The current study provides evidence for the prominent role of Tak1 in the proliferation of BMMSCs. Both in vivo and in vitro, it was confirmed that Tak1 deprivation leads to strong inhibition of the proliferative activity of BMMSCs without affecting their differentiation potential. Notably, 5zox treatment during differentiation suppressed chondrogenesis and osteogenesis, which was consistent with previous results observed in Tak1-suppressed cells [11, 12, 26, 27]. In the in vivo experiment, we observed that 5zox treatment blocked expansion of BMMSCs and induced growth retardation; however, both the number of BMMSCs and total body weights recovered to normal levels upon discontinuing the 5zox treatment. Although the toxicity of the 5zox is not well understood, it was shown that reversible forced blocking of cell cycling in BMMSCs was possible by inhibition of Tak1 activity. Subsequent analysis of the characteristics of the 5zox-treated cells showed that Tak1 inhibition resulted in the accumulation of $G_0/G_1$ cells.
Importantly, the 5zox-treated cells showed quiescent cell-specific characteristics in terms of gene expression and ROS resistance potential. Based on these findings, we concluded that Tak1 inhibition induced a quiescent-like state in the BMMSCs.

Originally, Tak1 was considered as a kinase activated by TGFβ [6–10, 28]. However, we observed that Tak1 is also activated by FGF2, EGF, and PDGFs. Previous studies have demonstrated that Tak1/TAK1 could be activated by various cytokines such as interleukins [7], FGFR1 [29], FGFR3 [30], G-CSF, and some chemotactic factors [31], in addition to TGFβ. Therefore, it was not surprising that Tak1 activation was induced by various cytokines tested in the present work. This Tak1 activation induced by various cytokines is thought to be an important factor engaging Tak1 as a hub molecule for the mitogenic reactions of BMMSCs.

To identify downstream molecules connecting Tak1 activity with the mitogenic reaction of BMMSCs, we performed inhibition of major molecules related to the self-renewal of stem cells. The involvement of Tak1 in the proliferation of neural stem cells [32], hematopoietic stem cells, nephron progenitor cells [33], and muscular satellite cells [16] has already been reported. In these cells, Tak1 is required for the activation of NF-κB and JNK during inflammation-induced tissue regeneration. However, we found that inhibition of NF-κB and JNK did not affect cell proliferation activity in BMMSCs, at least in the present TGFβ-dependent culture conditions. Consistent with our notion, it has previously been shown that inhibition of NF-κB signaling enables the generation of MSCs from pluripotent stem cells, rather than blocking their proliferation [34]. These findings suggested that NF-κB might be dispensable for MSC self-renewal. Several studies have shown the involvement of JNK in MSC proliferation; for example, JNK signaling is involved in FGF2- [35] and PDGF-induced proliferation [36], and treatment with SP600125 has been shown to inhibit the cytokine-induced proliferation. However, importantly, these studies also showed that JNK inhibition could not induce complete inhibition of cell proliferation, but only canceled the effect of cytokines.

It has been reported that Tak1 can also activate ERK and p38MAPK [37]. ERK is especially known as a strong mitogen in various cells, including stem cells. We observed that cell proliferation activity of BMMSCs was suppressed to 50% compared with that of the control. This result was consistent with that of siRNA experiment. Inhibition of p38MAPK or Akt also affected BMMSC proliferation, although the suppressive effects of p38MAPK and Akt inhibition on cell proliferation were not critical. Consistently, previous studies have shown that MSCs can repopulate under suppression of ERK [35], p38MAPK [38], and Akt [39].

In contrast to the above molecules, Tak1 inhibition with 5zox inhibited BMMSC proliferation almost completely, without affecting cell viability. This suggests that Tak1 plays a prominent role in the proliferation of BMMSCs, and its activity is likely mediated by a different mechanism from the well-studied models such as MAPKs.

In the context, we focused on Yap1/Taz, which fulfill the following requirements as a regulator of the BMMSC proliferation: abundantly expressed in various stem cells including BMMSCs [40], directly involved in cell proliferation, and can function independently of proliferation-related kinases such as ERK [41] and JNK [42].

In the nucleus, Yap1 and Taz form a complex with transcription factors, most prominently the TEA domain family members (TEADs), and strongly activate gene expression [43, 44] to induce cell proliferation. As expected, interaction between Tak1 and Yap1/Taz was observed in proliferating BMMSCs. Binding of Tak1 to Yap1/Taz was dependent on phosphorylation modification and dephosphorylation of Tak1 by 5zox treatment disrupted the protein–protein interaction between Tak1 and Yap1/Taz.

We then hypothesized that the interaction between Tak1 and Yap1/Taz regulates the degradation of Yap1/Taz. In previous studies, it has been demonstrated that Yap1 is negatively regulated by large tumor suppressor 1 (LATS1) and 2, which in turn phosphorylate Yap1 and induce recruitment of the ubiquitin ligase complex and subsequent degradation via the ubiquitin proteasome pathway [45–47]. Taz is also regulated by phosphorylation and subsequent ubiquitin-proteasomal degradation [24, 47].

Under 5zox treatment condition, we found that Yap1 phosphorylation on the Ser397 residue increased. Phosphorylation of YAP1Ser397 is modified by LATS and this induces recruitment of the SCF–β-TRCP E3 ubiquitin ligase, and leads to ubiquitylation and degradation of Yap1 [24, 48]. In mouse cells, phosphorylation on Ser397 residue and subsequent cytoplasmic retention and degradation were induced not only by Latz, but also by latz-independent Fak/Cdc42/Pp1a cascade [25]. Consistent with these findings, immunoblot with anti-Ubiquitin (Ub) antibodies showed that Yap1 was highly ubiquitinated under 5zox treatment condition. Although we could not observe the phosphorylation status of Taz because of antibody availability, an increase in ubiquitination-modification under 5zox treatment was also detected in Taz protein. From these, we concluded that Tak1 provided stability for Yap1 and TAZ by blocking the degradation marks and subsequent ubiquitination-proteasomal degradation of Yap1/Taz. In previous studies, Tak1 was reported to be additionally activated by mechanical stresses [49, 50]. Interestingly, the proliferation of BMMSCs is enhanced by fluid flow, fluid shear, and cyclic tensile strains. Furthermore, it has also been revealed that cyclic strain increases the activity of YAP1 [51]. Therefore, it is possible that the Tak1-Yap1 molecular axis demonstrated here serves as a common signaling cascade in mitogenic reactions in BMMSCs.

Until now, several studies suggest that induction of quiescence confers enhanced stress tolerance in somatic stem cells. As a result of the elevated expression of antistress molecules such as glutathione peroxidase 3 (GPX3), sulfiredoxin, and thioredoxin reductase 1 (TXNRD1), quiescent somatic stem cells are protected from various stress molecules including ROS [52]. It has been demonstrated that the induction of a quiescent state in human MSCs via serum deprivation enhances their viability when implanted in vivo [53].

To date, a variety of approaches to induce quiescence in vitro have been reported: anchorage deprivation, growth to confluence and contact inhibition, mitogen deprivation [53], and nutrient/amino acid limitation [54]. Although these approaches result in reversible quiescence in somatic cells and stem cells, the efficiency of these treatments is highly dependent on the condition and quality of the cells, and they sometimes lead to irreversible changes. For example, the blockade of cell adhesion induces anoikis in BMMSCs [55], and serum or mitogen deprivation leads to premature aging [56] or cell death [57]. Recently, it was reported that quiescence may be triggered by culture on soft substrates [58, 59]. However, this method needs complicated processes to induce reentry into active cell cycling status. Thus, alternative approaches to establish reversible quiescence in MSCs are preferable for actual therapeutic use. As expected, pretreatment with 5zox clearly improved survival of the transplanted BMMSCs.
in the muscle injury models. The 5zox pretreated BMMSCs showed lower ROS levels compared with untreated cells from the transplanted site, indicating that 5zox treatment provides stress tolerance, at least for oxidative stress, also in vivo and contributes to the higher survival rate of the transplanted cells. It is known that transplanted MSCs are hardly engrained when the cells are heterotopically transplanted [60]. To observe the effect of 5zox treatment on engrainment of BMMSCs, we performed IBMT of the BMMSCs, expecting that the original environment of the BMMSCs provide longer engrainment. Approximately, 1% cells (untreated) remained in the BM tissues on day 28 after transplantation. Notably, the 5zox treatment improved the engrainment rate of the BMMSCs; 5% of the transplanted cells remained on day 28 after transplantation. Furthermore, 40% of the EGFP+ fraction maintained PDGFβR and Sca1 expression even on day 28 after transplantation when the BMMSCs were pretreated with 5zox. These findings indicate that 5zox treatment not only improved survival and engrainment rate of BMMSCs, but also contributed to the maintenance of stem cell characteristics of the transplanted BMMSCs in the recipients. Interestingly, the microarray analysis and subsequent qRT-PCR-based validation suggested that 5zox treatment enhances gene expression of various cytokines involved in wound healing and immunomodulation, which are important therapeutic role of BMMSCs (Supporting Information Fig. S9). Although detailed analysis such as confirmation of protein expression is essential, it is possible that the improved cell survival and engrainment observed in the 5zox pretreated cells were supported through the expression of these cytokines. We believe that further characterization of the 5zox-treated and -converting stem cell self-renewal, but also for improving the therapeutic effect of BMMSCs.

CONCLUSION

In the present study, we show that Tak1 is a crucial molecule for the activation of cell cycling in BMMSCs. Inhibition of Tak1 leads to synchronization in quiescence both in vitro and in vivo. Tak1 can be activated by various growth factors, and, in turn, it activates cell cycling of BMMSCs through stabilization of Yap1/Taz. The quiescent induction by Tak1 inhibition provides stress tolerance and improved engrainment properties to the BMMSCs. Thus, Tak1 is an important molecule not only for clarifying stem cell self-renewal, but also for improving the therapeutic effect of BMMSCs.

REFERENCES

1. Andrzejewska A, Lukomska B, Janowski M. Mesenchymal stem cells: From roots to boost. Stem Cells 2019;37:855–864.
2. Naji A, Favier B, Deschaseaux F et al. Mesenchymal stem/stromal cell function in modulating cell death. Stem Cell Res Ther 2019;10:56.
3. Ding W, Knox TR, Tschumper RC et al. Platelet-derived growth factor (PDGF)-PDGF receptor interaction activates bone marrow-derived mesenchymal stromal cells derived from chronic lymphocytic leukemia: Implications for an angiogenic switch. Blood 2010;116:2984–2993.
4. Ng F, Boucher S, Koh S et al. PDGF, TGF-β, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): Transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. Blood 2009;112:2984–2993.
5. Zhang YE. Non-Smad pathways in TGF-beta signaling. Cell Res 2009;19:128–139.
6. Ninomiya-Tsujii J, Kishimoto K, Hyama A et al. The kinase TAK1 can activate the NIK-1 kappaB as well as the MAP kinase cascade in the IL-1 signaling pathway. Nature 1999;398:252–256.
7. Wan Y, Chi H, Xie M et al. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol 2006;7:851–858.
8. Stockert J, Wolf A, Kaddatz K et al. Regulation of TAK1/TAB1-mediated IL-1beta signaling by cytoplasmic PPARbeta/delta. PLoS One 2013;8:e63011.
9. Shim JH, Greenblatt MB, Xie M et al. TAK1 is an essential regulator of BMP signaling in cartilage. EMBO J 2009;28:2028–2041.
10. Shinohara H, Nagashima T, Cascalho MI et al. TAK1 maintains the survival of immunoglobulin lambda-chain-positive B cells. Genes Cells 2016;21:1233–1243.
11. Gunnell LM, Jonson JH, Loiselle AE et al. TAK1 regulates cartilage and joint development via the MAPK and BMP signaling pathways. J Bone Miner Res 2010;25:1784–1797.
12. Gao L, Sheu TJ, Dong Y et al. TAK1 regulates SOX9 expression in chondrocytes and is essential for postnatal development of the growth plate and articular cartilages. J Cell Sci 2013;126:5704–5713.
13. Song Z, Liu C, IWata J et al. Mice with Tak1 deficiency in neural crest lineage exhibit cleft palate associated with abnormal tongue development. J Biol Chem 2013;288:10440–10450.
14. van Beuningen HM, de Vries-van Melle ML, Vitters EL et al. Inhibition of TAK1 and/or JAK can rescue impaired chondrogenic differentiation of human mesenchymal stem cells in osteoarthritis-like conditions. Tissue Eng Part A 2014;20:2243–2252.
15. Hoffmann A, Preobrazhenska O, Wodarczyk C et al. Transforming growth factor-beta-activated kinase-1 (TAK1), a MAP3K, interacts with Smad proteins and interferes with osteogenesis in murine mesenchymal progenitors. J Biol Chem 2005;280:27271–27283.
16. Ogura Y, Hidari Y, Sato S et al. TAK1 modulates satellite stem cell homeostasis and skeletal muscle repair. Nat Commun 2015;6:10123.
17. Zhu H, Guo ZK, Jiang XK et al. A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. Nat Protoc 2010;5:550–560.
18. Sakaguchi Y, Sekiya I, Yagishita K et al. Comparison of human stem cells derived from various mesenchymal tissues: Superiority of
Note 1: synovium as a cell source. Arthritis Rheum 2005;52:2521–2529.

19 Sakaue-Sawano A, Kurokawa H, Morimura T et al. Visualizing spatiotemporal dynamics of multcellular cell-cycle progression. Cell 2008;132:487–498.

20 Zielke N, Edgar BA. Fucci sensors: Powerful new tools for analysis of cell proliferation. Wiley Interdiscip Rev Dev Biol 2015;4:469–487.

21 Kushida T, Inaba M, Hisha H et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001;97:3292–3299.

22 Nakamura K, Inaba M, Sugira K et al. Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of GVHD by intra-bone marrow bone marrow transplantation plus donor lymphocyte infusion. Stem Cells 2004;22:125–134.

23 Ninomiya-Tsuji J, Kajino T, Ono K et al. A resorcylic acid lactone, 5Z-7-ozoneaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase. J Biol Chem 2003;278:18480–18490.

24 Liu CY, Zha ZY, Zhou X et al. The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphoryode and recruiting the SCF(beta-TrCP) E3 ligase. J Biol Chem 2010;285:37159–37169.

25 Hu JK, Du W, Shelton SJ et al. An FAK-mTOR signaling axis regulates stem cell-based tissue renewal in mice. Cell Stem Cell 2017;21:91:e106–e106.

26 Yang H, Guo Y, Wang D et al. Effect of TAK1 on osteogenic differentiation of mesenchymal stem cells by regulating BMP-2 via Wnt/beta-catenin and MAPK pathway. Organogenesis 2018;14:36–45.

27 Zhang Y, O’Keefe RJ, Jonason JH. BMP TAK1 (MAP3K7) induces adipocyte differentiation through PPARgamma signaling. J Cell Biochem 2017;118:204–210.

28 Yamaguchi K, Shirakabe K, Shibuya H et al. Identification of a member of the MAPKK family as a potential mediator of TGF-beta signal transduction. Science 1995;270:2008–2011.

29 Wang C, Ke Y, Liu S et al. Ectopic fibroblast growth factor receptor 1 promotes inflammation by promoting nuclear factor-kappaB signaling in prostate cancer cells. J Biol Chem 2018;293:14839–14849.

30 Salazar L, Kashiwada T, Krejci P et al. Fibroblast growth factor receptor 3 interacts with and activates TGFbeta-activated kinase 1 tyrosine phosphorylation and NF-kappaB signaling in multiple myeloma and bladder cancer. PLoS One 2014;9:e86470.

31 Sylvain-Prevost S, Ear T, Simard FA et al. Activation of TAK1 by chemotactic and growth factors, and its impact on human neutrophil signaling and functional responses. J Immunol 2015;195:5393–5403.