injection of excess anesthetic, and the implants were harvested. The harvested implants were fixed with 10% neutral buffered formalin, and decalcified sections were stained with hematoxylin and eosin. Bone formation was confirmed by immunohistochemistry using a primary antibody against osteocalcin (1:5000, OCG3, Takara Bio). Because the mineral densities of the woven bone and β-TCP were very similar, the bone tissue could not be distinguished from the scaffold clearly using micro computed tomography. Therefore, we quantified the bone tissue in the porous β-TCP blocks using histological images [29, 34]. Three sections at equal intervals were prepared from one implant and stained with hematoxylin and eosin. Then, bone tissues were selected manually and bone formation was quantified using imaging software. The bone formation area of one implant was determined from the average value of three sections.

### Statistical analysis

Real-time PCR data were analyzed using a two-way repeated-measures analysis of variance (ANOVA), and multiple comparisons were performed using the Bonferroni correction. The data from the CFU assay were analyzed using Student's t-test. The bone formation ratio was analyzed using a one-way ANOVA. As a post-hoc test, multiple comparisons between groups were performed using Student's t-test with the Bonferroni correction.

## Results

### 1. Dexamethasone alters the proliferation of bone marrow-derived and muscle-derived stromal cells

The BMSCs cultured in the control medium proliferated until 21 days of culture, after which the proliferation rate decreased rapidly. The lowest concentration of dexamethasone ($10^{-8}$M) augmented the proliferation of the BMSCs throughout the experimental period. The proliferation rates of the BMSCs treated with $10^{-7}$ and $10^{-6}$ M dexamethasone were lower than those of untreated cells at the early stage of culture. However, the proliferation rates did not decrease during the culture period, in contrast to the proliferation of the untreated BMSCs. Furthermore, the BMSCs treated with $10^{-7}$ and $10^{-6}$ M dexamethasone showed similar proliferation (Fig. 1A). MuSCs without dexamethasone treatment proliferated at a much higher rate than dexamethasone-treated MuSCs throughout the culture period. Dexamethasone-treated MuSCs proliferated at much lower rates than cells cultured without dexamethasone. In particular, the proliferation of MuSCs cultured in $10^{-7}$ and $10^{-6}$ M dexamethasone was strongly suppressed to similar levels (Fig. 1B). These results indicate that dexamethasone strongly suppresses the proliferation of BMSCs and MuSCs, particularly MuSCs.

### 2. Dexamethasone pretreatment and osteogenic induction by the combination of dexamethasone and BMP-2 enhance the osteogenic differentiation of BMSCs and MuSCs

Osteogenic induction was performed as shown in Fig. 2A. Preliminary studies confirmed that in both BMSCs and MuSCs, cells treated with $10^{-7}$ and $10^{-6}$ M dexamethasone showed the highest osteogenic differentiation, and there was no significant difference between the concentrations (data not shown). Therefore, in subsequent studies, we compared cells cultured in the control medium and cells cultured in the medium containing $10^{-7}$ M dexamethasone. ALP staining and Von Kossa staining were used to determine the ALP activity and mineralization capability of the BMSCs (BM and BM-Dex cells) (Fig. 2B), and mRNA expression of osteogenic markers was also evaluated (Fig. 2C). All of these assays showed that the osteogenic capacity of BM-Dex cells was remarkably higher than that of BM cells ($P<0.05$). BM-Dex-AGD cells

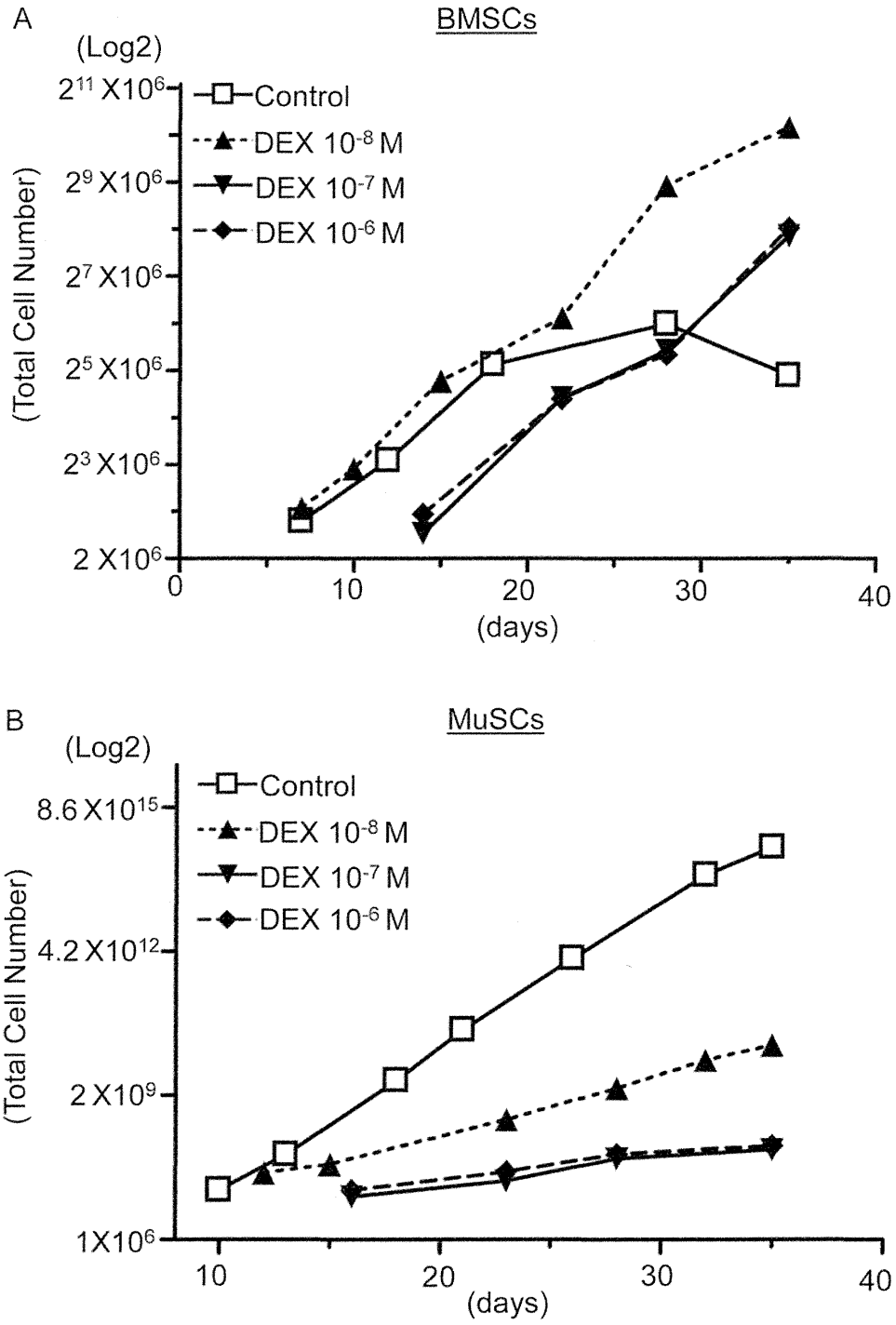


**Figure 1. Alteration of proliferation of BMSCs and MuSCs cultured with dexamethasone.** A: Proliferation rate of BMSCs cultured with $10^{-8}$ M, $10^{-7}$ M, or $10^{-6}$ M dexamethasone or without dexamethasone for 35 days. Total cell numbers (Y-axis) are expressed using a log2 scale. B: Proliferation rate of MuSCs cultured with $10^{-8}$ M, $10^{-7}$ M, or $10^{-6}$ M dexamethasone or without dexamethasone for 35 days. Total cell numbers (Y-axis) are expressed using a log2 scale.

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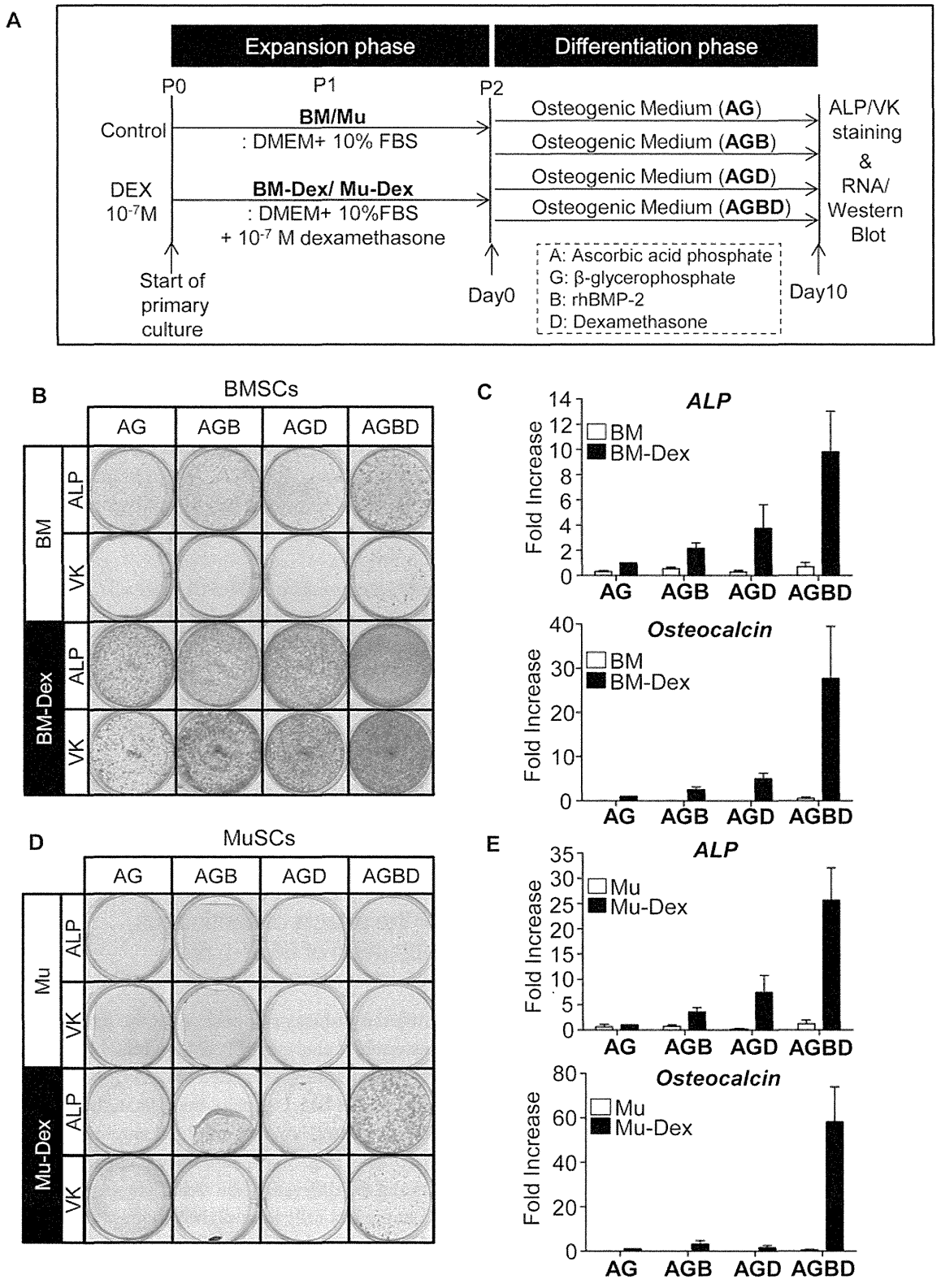


**Figure 2. Dexamethasone pretreatment and osteogenic induction with combined dexamethasone and BMP-2 treatment enhance the osteogenic differentiation of BMSCs and MuSCs.** A: Schematic representation of the cell culture protocol. Gross images of ALP staining (ALP) and Von Kossa staining (VK) of BMSCs (B) and MuSCs (D). Quantitative analysis of the mRNA expression of ALP and osteocalcin in BMSCs (C) and MuSCs (E). The fold change in gene expression was normalized to that of BM-Dex-AG or Mu-Dex-AG. Bars show the mean and SEM. Statistical significance was confirmed between the

BM and BM-Dex groups (ALP: p = 0.015, OCN: p = 0.023) and between the Mu and Mu-DEX groups (ALP: p = 0.019, OCN: p = 0.015). Effects of combinations of differentiation reagents were significant for ALP in BM-Dex (p = 0.032) and Mu-DEX (p = 0.019) and for OCN in Mu-DEX (p = 0.024).

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showed significantly higher ALP and osteocalcin mRNA expression than BM-Dex-AG cells, which were treated with dexamethasone during expansion but not treated with dexamethasone during osteogenic induction. A comparison between BM-Dex-AGB and BM-Dex-AGBD cells revealed that inclusion of dexamethasone during osteogenesis enhanced the effects of BMP-2 on osteogenesis. Among all of the groups, BM-Dex-AGBD cells presented the strongest ALP and Von Kossa staining, with concomitantly high ALP and osteocalcin mRNA expression. The BM-AGBD cells presented the highest ALP and osteocalcin mRNA expression among BM cells, although the expression levels were still far lower than those in BM-Dex cells.

Among Mu cells, although low mRNA expression of ALP was detected, all of the wells were almost negative for ALP staining, and no calcified nodules were detectable on Von Kossa staining (Fig. 2D and 2E). Conversely, the wells containing Mu-Dex cells presented scattered ALP-positive colonies, and the ALP staining intensity of each positive colony was highest in wells containing Mu-Dex-AGBD cells. Von Kossa staining of Mu-Dex-AGBD cells clearly demonstrated mineralized nodules, which confirmed that Mu-Dex cells are capable of mineralization. The scattered nature of the staining indicated that cells with osteogenic capability comprised only a small portion of the Mu-Dex cells. ALP mRNA expression was significantly higher in Mu-Dex-AGD and Mu-Dex-AGBD cells than in Mu-Dex-AG and Mu-Dex-AGB cells, respectively, indicating that dexamethasone promotes ALP expression in MuSCs. Osteocalcin mRNA expression in Mu-Dex cells without BMP-2 (Mu-Dex-AG and AGD cells) was significantly lower than that in Mu-Dex cells with BMP-2 treatment (Mu-Dex-AGB and AGBD cells). Among the BMP-2-treated Mu-Dex cells, Mu-Dex-AGBD cells showed significantly higher osteocalcin mRNA expression than Mu-Dex-AGB cells differentiated without dexamethasone. These findings suggest that BMP-2 plays an important role in the late stage of osteogenic differentiation of MuSCs and that its effects are enhanced by dexamethasone treatment. Thus, dexamethasone treatment throughout the culture period is critical for osteogenesis and dexamethasone enhances osteogenic differentiation with or without application of BMP-2 in both bone marrow-derived and muscle-derived stromal cells.

## 3. SMAD signaling is responsible for the effects of continuous dexamethasone treatment and combination of BMP-2 and dexamethasone

We examined BMP signaling pathways to identify the pathway responsible for the interactive effect between BMP-2 and dexamethasone. Western blot analyses of BMSCs after 24 h of osteogenic induction showed significantly increased levels of phosphorylated SMAD (P-SMAD) 1/5 in the BM-Dex cells compared to the BM cells. In the BM-Dex cells, the cells in the AGB, AGD, and AGBD conditions showed clear P-SMAD expression, and among them, the cells in the AGBD condition showed the highest P-SMAD level (Fig. 3A). There was no apparent difference in non-phosphorylated SMAD levels among the differentiation treatment groups in both BM and BM-Dex cells, although the levels in the BM cells were slightly higher than those in the BM-Dex cells. This finding indicates that the BM-Dex cells, which were expanded with continuous dexamethasone treatment, had significantly higher reactivity to BMP stimulation.

Western blot analyses of MuSCs showed results similar to those of BMSCs, i.e., increased levels of P-SMAD in the Mu-Dex-AGB and AGBD cells and a significantly higher level in Mu-Dex-AGBD cells than in Mu-Dex-AGB cells. In contrast to Mu-Dex cells, Mu-AGB and

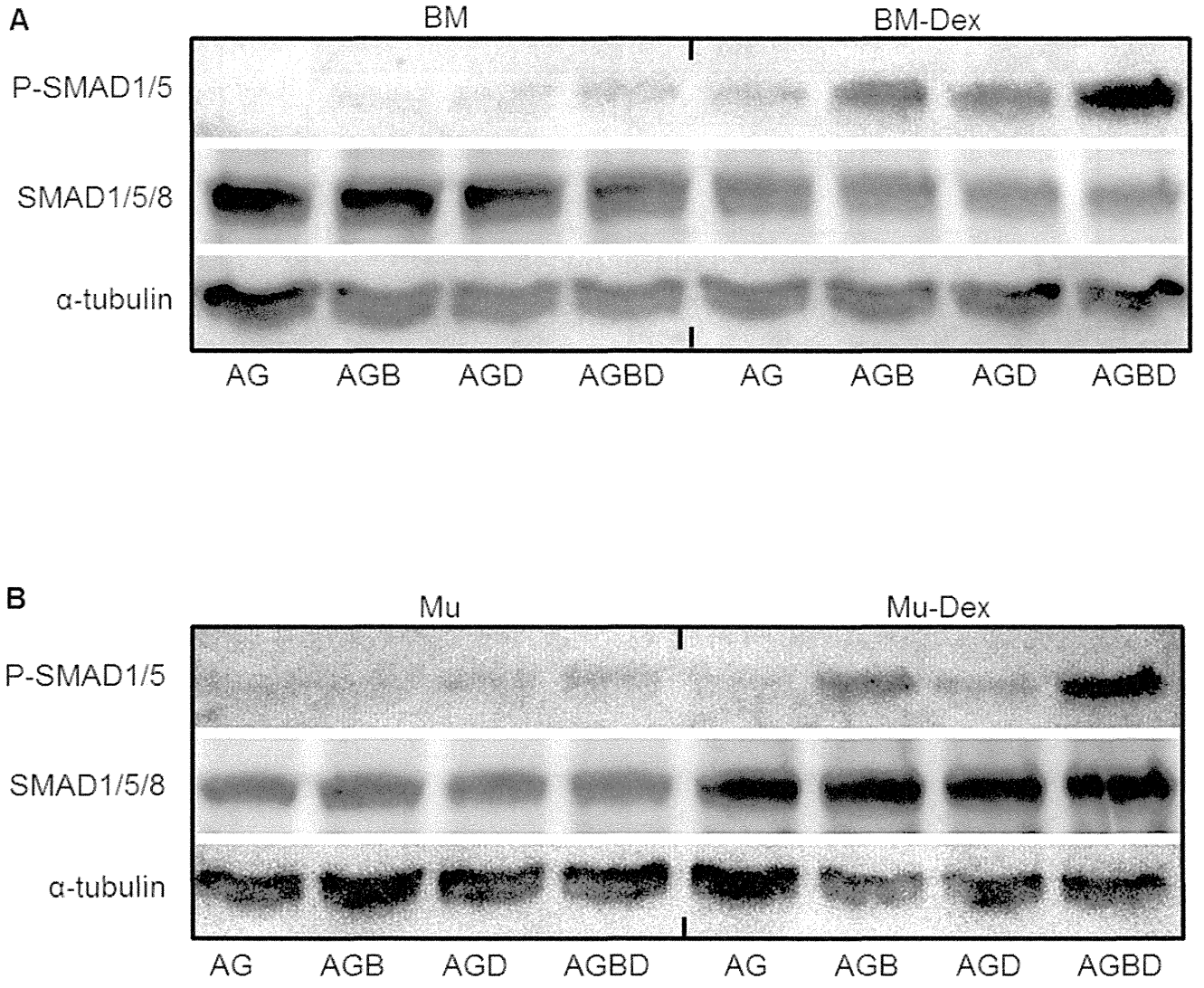


**Figure 3. Western blot analyses of the SMAD1/5/8 and phosphorylation of SMAD 1/5.** Western blot analyses of P-SMAD 1/5, SMAD1/5/8 and α-tubulin expression in BMSCs (A) and MuSCs (B) under four different osteogenic induction conditions with or without dexamethasone.

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AGBD cells showed only a slight increase of P-SMAD levels. Among MuSCs, non-phosphorylated basal SMAD levels also increased in the BMP-treated groups (Fig. 3B).

Based on these results, the BMP-SMAD signaling pathway contributed to the augmentation of osteogenic differentiation in the BM-Dex and Mu-Dex groups compared with the BM and Mu groups, and further contributed to augmentation of osteogenic differentiation in the AGBD treatment groups.

## 4. Dexamethasone treatment alters the subpopulations of bone marrow-derived cells and muscle tissue-derived cells

To investigate the effects of dexamethasone on subpopulations of BMSCs and MuSCs, which are both heterogeneous cell populations, colony-forming unit (CFU) assays were conducted as shown in Fig. 4A. The BM cells (ND- ), which had not been treated with dexamethasone, and

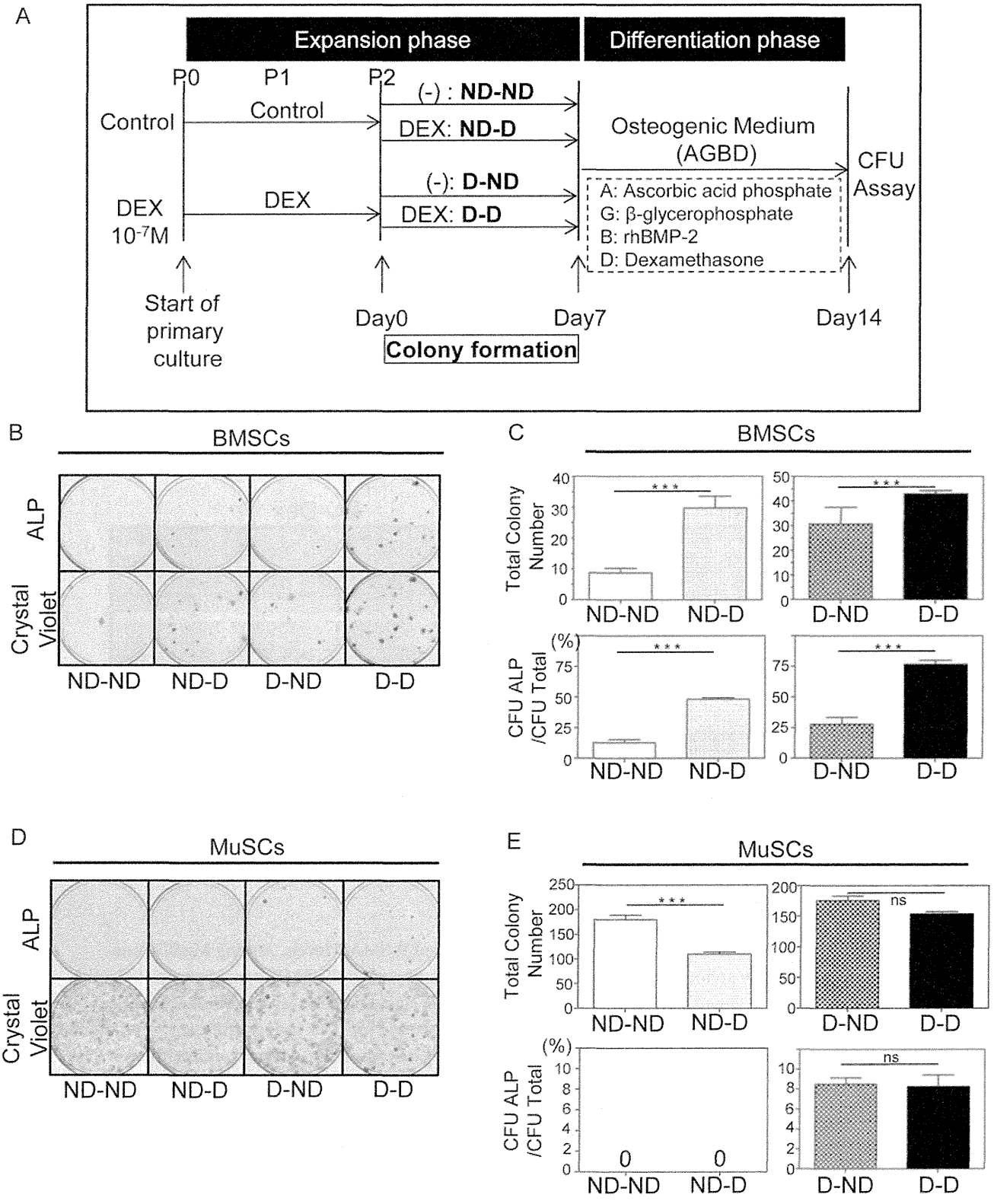


**Figure 4. Colony formation assay of BMSCs and MuSCs.** A: Schematic representation of the colony formation unit assay protocol. Cells seeded at P2 were allowed to form single-cell-derived colonies with or without $10^{-7}$ M dexamethasone for 7 days before osteogenic induction. ND-ND indicates normal growth medium at P0, P1, and P2. ND-D indicates normal growth medium at P0 and P1 and dexamethasone-containing medium at P2. D-ND indicates dexamethasone-containing medium at P0 and P1 and normal growth medium at P2. D-D indicates dexamethasone-containing medium at P0, P1, and P2.

Gross images of BMSCs (B) and MuSCs (D) in each dish stained by ALP and crystal violet. Quantification of the total colony number and fraction of ALP-positive colonies (%) among total colonies in BMSCs (C) and MuSCs (E). *** denotes $P < 0.001$ as determined by Student's t-test.

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BM-Dex cells (D- ), which had been exposed to dexamethasone through the culture period, were allowed to form single-cell-derived colonies with (-D) or without dexamethasone (-ND) for 7 days and then cultured in osteogenic induction medium for another 7 days. In BM cells, the ALP-positive colony ratio and total colony number were significantly higher in cultures treated with dexamethasone during colony formation (ND-D) than in those not treated with dexamethasone (ND-ND) (Fig. 4B and 4C), which indicates that dexamethasone treatment during colony formation selectively promoted the proliferation of specific subpopulations with osteogenic capability that had been contained in the BM cells and that had not been able to proliferate without dexamethasone. In BM-Dex cells, not only the ratio of ALP-positive colonies but also the total colony number was decreased by withdrawal of dexamethasone in D-ND compared to D-D, in which the cells were exposed to dexamethasone throughout the culture period (Fig. 4B and 4C). This finding indicates that some cell subpopulations that required dexamethasone to proliferate and form colonies were contained in BM-Dex cells and a part of such populations could not form colonies on withdrawal of dexamethasone. Therefore, dexamethasone may selectively promote the proliferation of cells with osteogenic potential and simultaneously suppress the proliferation of cells without differentiation potential.

Selective effects of dexamethasone were also observed in MuSCs (Fig. 4D and 4E). However, the Mu cells used in the CFU assay had been cultured in the control medium and showed extensive proliferation for 14 days without selective effects of dexamethasone. Therefore, nearly all of these cells already lacked osteogenic capability, and the ALP-positive ratio was almost 0% despite the dexamethasone treatment during colony formation. In contrast, Mu-Dex cells cultured in dexamethasone-containing medium from the beginning of the culture demonstrated an ALP-positive ratio of nearly 10% regardless of the treatment applied during the colony formation period. These results indicate that dexamethasone treatment alters the proliferation of subpopulations of BMSCs and MuSCs, resulting in an increased ratio of cells with osteogenic potential.

## 5. Dexamethasone affects cell proliferation during osteogenic differentiation

Cell proliferation during osteogenic differentiation was analyzed by quantifying the amount of dye bound to the cells. The BM and BM-Dex cells in each osteogenic differentiation condition proliferated during differentiation. The BM cells guided to differentiate in the absence of dexamethasone (BM-AG and BM-AGB cells) proliferated faster than BM cells differentiated in the presence of dexamethasone (BM-AGD and BM-AGBD cells) in the early stage of differentiation, and then this pattern reversed as differentiation progressed (Fig. 5A). No significant differences in proliferation rates were observed among BM-Dex cells at the early stage of differentiation (Fig. 5B). However, the proliferation rates of BM-Dex-AG and BM-Dex-AGB cells declined at the later stage of differentiation relative to those of BM-Dex-AGD and AGBD cells cultured in dexamethasone.

In MuSCs, dexamethasone remarkably suppressed the proliferation of both Mu and Mu-Dex cells during osteogenic differentiation (Fig. 5C and 5D). Mu-Dex-AG and Mu-Dex-AGB cells, which showed slow proliferation in dexamethasone-containing medium during expansion culture, showed rapid proliferation in dexamethasone-free medium. These results

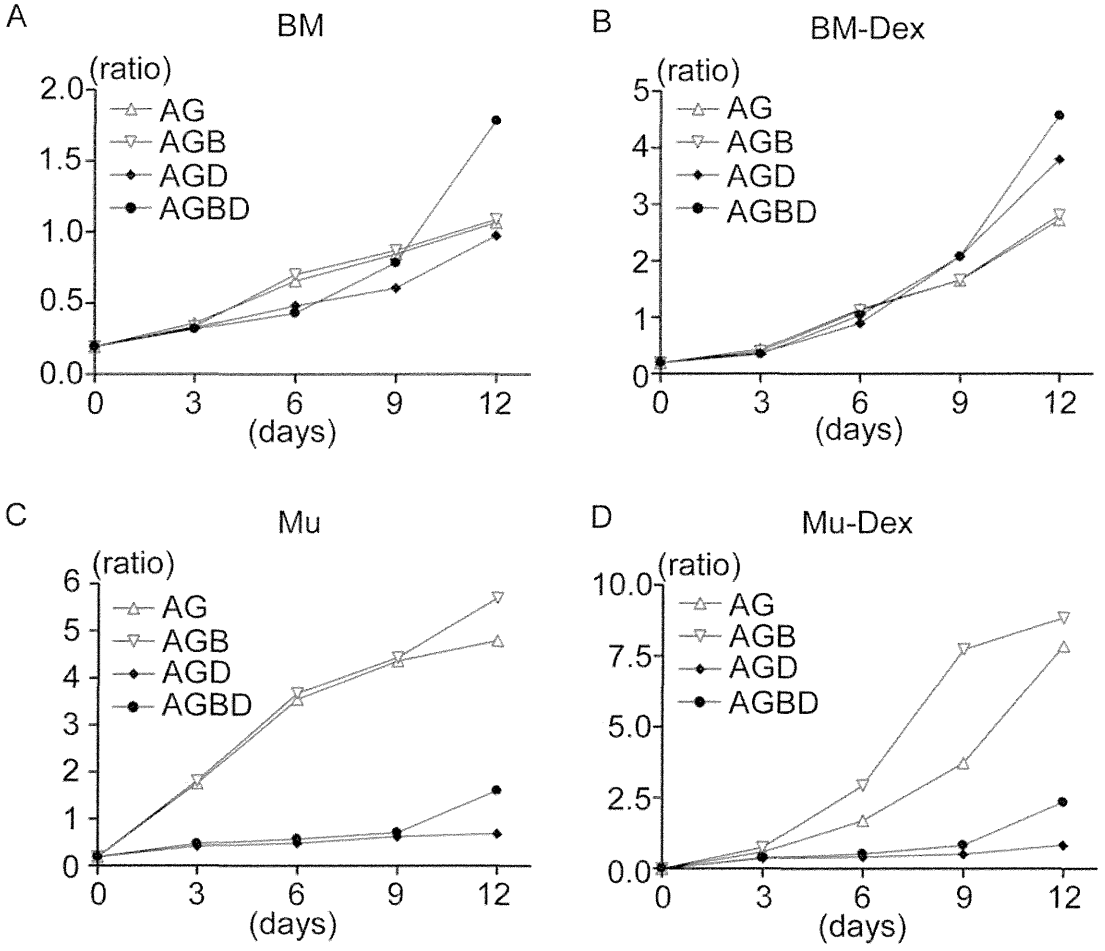


**Figure 5. Dexamethasone affects cell proliferation during osteogenic differentiation.** The absorbance at 585 nm was measured for dye extracted from the wells, and ratios relative to the standard are presented in the graphs. A: BM, B: BM-Dex, C: Mu, and D: Mu-Dex

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were consistent those of the cell proliferation assay and indicated that dexamethasone also affects the proliferation and subpopulation composition of BMSCs and MuSCs during differentiation.

## 6. Bone formation capability of bone marrow and muscle-derived cells

To confirm the bone formation capability of BMSCs and MuSCs, porous β-TCP blocks loaded with bone marrow and muscle-derived cells that had been expanded with or without dexamethasone and with or without BMP-2 were subcutaneously transplanted into rats. After 4 weeks, the implants were harvested and histologically examined (Fig. 6A). In the rats treated with BMSCs, abundant bone formation was observed in every treatment condition. In the rats treated with MuSCs, bone tissue was identified in all four blocks of the group that received cells cultured with both dexamethasone and BMP-2, although the bone tissue occupied only a small portion of each implant. No bone tissue was observed in the blocks of the other groups. This result was consistent with the in vitro mineralization results in cultured MuSCs and confirmed the bone formation capability of muscle-derived cells.

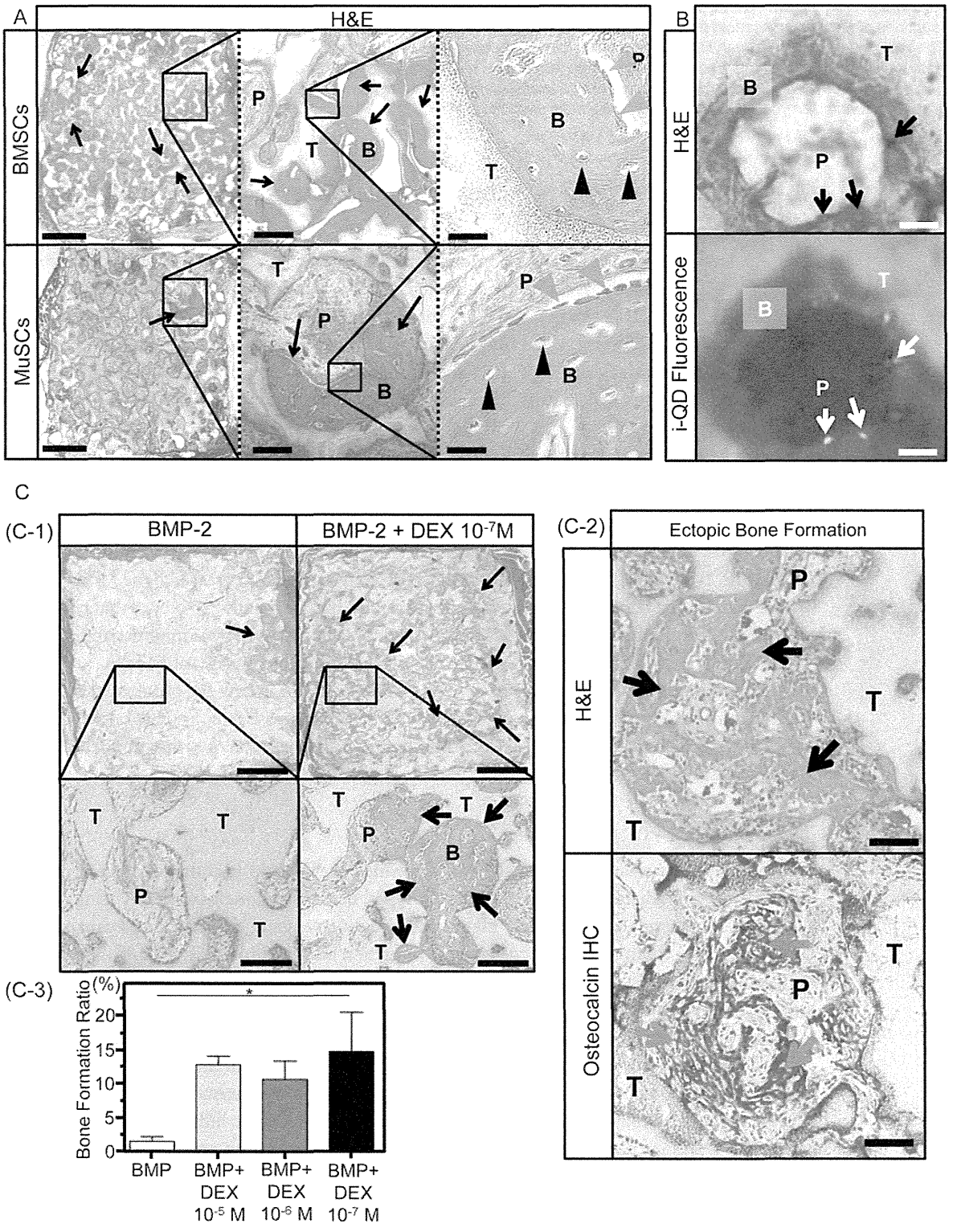


**Figure 6. Ectopic bone formation analyses.** A: Bone formation capability of muscle-derived cells. Representative histological sections of a scaffold loaded with BMSCs or MuSCs cultured with both dexamethasone and BMP-2. Scale bar: 1 mm (left panels), 200 μm (middle panels) and 50 μm (right panels). Black arrows indicate new bone formation in the scaffold. Black arrow heads indicate osteocytes and green arrow heads indicate bone lining cells. B: Newly formed bone, T: β-TCP, P: Porous area. B: Recruitment of cells residing in muscle tissue to participate in BMP-2-induced ectopic bone formation. Cells labeled prior to local BMP-2 administration were detected in the newly formed woven bone area. Representative histological sections were stained with H&E and evaluated for i-QD fluorescence. The black arrows in the H&E image show the locations of fluorescently labeled cells indicated with white arrows in the

fluorescent image. Scale bar: 200 μm. B: Newly formed bone, T: β-TCP, P: Porous area. C: Augmentation of ectopic bone formation by dexamethasone. C-1, 2: Representative histological sections of an excised scaffold that had been loaded with BMP-2 alone and a scaffold that had been loaded with dexamethasone and BMP-2. The sections were stained with H&E and immunostained for osteocalcin. Black arrows indicate new bone formation in the scaffold. Red arrows indicate osteocalcin positive staining area. Scale bar: 1 mm (top panels of C-1), 200 μm (bottom panels of C-1) and 50 μm (C-2). B: Newly formed bone, T: β-TCP, P: Porous area. C-3: Quantification of bone formation at 3 weeks after transplantation. The Y axis indicates the bone formation ratio calculated as total bone area/total scaffold area. Each bar represents the mean with the standard deviation (SD). *denotes $P < 0.05$.

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### 7. Recruitment of cells residing in muscle tissue for ectopic bone formation induced by BMP-2

It is well known that BMP-2 injected into muscle tissue induces bone formation at the administration site. To characterize the cells recruited to BMP-2-administered sites for heterotopic bone formation, i-QDs were injected into muscle tissue prior to local BMP-2 administration. Fluorescence microscopy revealed the presence of i-QD-labeled cells among bone-forming osteoblasts in the muscle tissue (Fig. 6B), which indicates that endogenous cells in the muscle tissue were recruited to participate in heterotopic bone formation induced by BMP-2.

### 8. Augmentation of ectopic bone formation by dexamethasone

Based on these results, we determined whether dexamethasone augments bone formation induced by BMP-2 in vivo. Fig. 6C-1 presents representative histological sections from an excised β-TCP block that had been loaded with BMP-2 and a block that had been loaded with both BMP-2 and 0.031 ng of dexamethasone. Histology indicated increased bone formation in β-TCP blocks that contained both dexamethasone and BMP-2 relative to blocks that contained only BMP-2, and the formed tissue was confirmed to be bone tissue by immunostaining of osteocalcin (Fig. 6C-2). The area of the formed heterotopic bone was quantified (Fig. 6C-3). There was no significant difference in the area of formed bone among the groups treated with dexamethasone; however, the area of formed bone in the groups treated with dexamethasone was significantly higher than that in the group treated with BMP-2 alone, thus confirming that dexamethasone also augments the osteogenic activity of BMP in vivo.

## Discussion

In this study, we evaluated BMSCs and MuSCs, which are thought to contribute to ectopic bone formation induced by BMPs. In both cell types, dexamethasone treatment during expansion culture resulted in considerably higher subsequent osteogenic differentiation capability. Dexamethasone treatment during osteogenic induction also promoted osteogenic differentiation induced both with and without BMP-2, and the combination of dexamethasone and BMP-2 had the strongest effect on osteogenic differentiation in both BMSCs and MuSCs.

The proliferation study and CFU assay revealed that dexamethasone differentially affected the proliferation and composition of cell subpopulations, resulting in the selection of cells with higher osteogenic capability among both BMSCs and MuSCs. Furthermore, dexamethasone treatment had a similar effect on cell proliferation during osteogenic induction, which suggests that dexamethasone also exerts selective effects on cell subpopulation composition during differentiation. Based on these results, we confirmed that subpopulation selection is at least one of the mechanisms by which dexamethasone augments the osteogenic differentiation of BMSCs and MuSCs, not only during expansion but also during osteogenic induction. The western blot analyses indicated augmented BMP-SMAD signaling in BMSCs and MuSCs expanded with dexamethasone compared to those expanded without dexamethasone. Therefore, we speculate that cells that had higher responsiveness to BMP stimulation selectively proliferated under continuous dexamethasone treatment. However, among the differentiation treatment groups, the

combination of BMP and Dex in AGBD cells resulted in a significantly higher level of P-SMAD than that observed in AGB and AGD cells. This finding suggests that mechanisms other than selective proliferation may be involved in the enhanced osteogenic differentiation observed for Dex, particularly because the differences were evident within 24 h of the treatments.

Many studies using BMSCs obtained by primary culture have shown effects of dexamethasone on not only osteogenic differentiation but also chondrogenic and adipogenic differentiation. However, the mechanisms underlying these effects have not been clarified. Some studies have evaluated the effects of dexamethasone on osteoblastic or progenitor cell lines established from not only rodents but also humans. However, most of these studies aimed to clarify the mechanisms underlying steroid-induced osteoporosis [38–44]; furthermore, the cell lines evaluated were homogenous and the findings may thus be difficult to generalize to physiologically or clinically relevant cell populations. Dexamethasone has not been used for osteogenic induction in most cell lines, even though it has been shown that dexamethasone is required for osteogenic induction of heterogeneous bone marrow-derived cells and cells from other stromal tissues. Therefore, we focused on the effects of dexamethasone on heterogeneous BMSC populations. The findings in this study agreed with our previous findings that human BMSCs treated with dexamethasone during proliferation presented enhanced osteogenic, chondrogenic, and adipogenic differentiation [29]. To date, many studies have characterized the osteogenesis of BMSCs using dexamethasone. However, most of those studies did not account for the heterogeneity of the cells, and few reports have directly indicated a selective effect of dexamethasone on BMSC subpopulations such as that shown in the present study. Moreover, this is the first study to report effects of dexamethasone on MuSC proliferation. Aubin and colleagues extensively investigated the effects of dexamethasone on various types of stromal cells. They reported that dexamethasone redistributed the subpopulations of fetal rat calvaria-derived cells, resulting in enhanced osteogenic [45], chondrogenic [46], and adipogenic differentiation [47]. They also studied effects of dexamethasone on subpopulations of rat BMSCs and demonstrated that dexamethasone significantly increased subpopulations with ALP positivity and bone nodule formation [48]. Furthermore, they studied the effects of dexamethasone on rat BMSCs and reported that BMSCs contained various subpopulations. BMSCs at the early stages of culture contained subpopulations that formed bone nodules without dexamethasone treatment, and such subpopulations decreased as the culture duration increased. Dexamethasone altered the composition of the subpopulations and increased the subpopulations of BMSCs that required dexamethasone to form bone nodules. Furthermore, Aubin and colleagues indicated that some subpopulations contained in BMSCs inhibited osteogenic differentiation and that dexamethasone may affect differentiation indirectly through such subpopulations [49]. Some reports have also indicated that the timing of dexamethasone treatment is important, i.e., that treatment at the early stage of primary culture has a stronger effect on osteogenic differentiation [50]. Such results also support a selective effect of dexamethasone on cell subpopulations, as the results were obtained during the early proliferative phase, when cell selection by competitive proliferation is likely to occur. However, as mentioned above regarding the western blot analysis, such population-selective effects of dexamethasone cannot completely explain the observed enhancement of osteogenesis. In this regard, we have also confirmed that dexamethasone augments the osteogenic differentiation of immortalized human BMSCs, which are considered to be a single-cell-derived and homogenous population because they have been passaged numerous times (data not published). Furthermore, Mikami et al. previously reported a synergistic effect of dexamethasone and BMP-2 in the C3H10T1/2 cell line [51]. These studies using homogenous cell populations may thus depict a different process than our proposed mechanism of cell subpopulation selection by competitive proliferation, which can only be studied in

a heterogeneous cell population such as that introduced by the present study. Although we could not completely clarify the effects of dexamethasone on the osteogenesis of stromal cells, we succeeded in demonstrating the subpopulation selection effect of dexamethasone, which can only be evaluated in heterogeneous cell populations, in addition to the dexamethasone-mediated augmentation of osteogenic differentiation induced by BMP-2. We consider that our results in heterogeneous cells obtained by primary culture are more applicable to in vivo conditions and clinical bone regeneration.

Regarding heterotopic bone formation induced by BMP-2 in muscle tissue, BMSCs, which have high osteogenic capability, and MuSCs, which reside around the implants, are expected to contribute to bone formation. Otsuru et al. directly confirmed that circulating bone marrow-derived progenitor cells differentiated into osteoblasts and formed bone tissue at muscle sites that received BMP-2-containing scaffolds in a mouse parabiotic pairing model [52]. Several previous studies demonstrated the contribution of muscle-derived cells to BMP-induced ectopic bone formation by transplanting muscle tissue-derived cells combined with BMP-2 into muscle [53, 54]. We also evaluated whether MuSCs contribute to heterotopic bone formation by BMP-2 and have bone formation capability. To identify the cells responsible for the ectopic bone formation, we labeled the cells in the muscle compartment recipient site prior to implantation. Fluorescently labeled cells were found at the site of ectopic bone formation, indicating that the labeled muscle cells migrated into the scaffold and differentiated into osteoblasts. We also performed a cell transplantation experiment to further evaluate the bone formation capability of MuSCs. Only MuSCs cultured with dexamethasone and BMP-2 showed bone formation in subcutaneous sites, although the area of the formed bone was limited. This finding simultaneously confirmed the bone formation capability of MuSCs and the effectiveness of the combination of dexamethasone and BMP-2 for MuSC-derived osteogenesis.

Previously, it was reported that systemic administration of dexamethasone enhanced ectopic bone formation by BMP-7 in murine muscles. In particular, implantation of a dexamethasone pellet into subcutaneous tissue increased the volume of ectopic bone induced by BMP-7 by 102% at 20 days of BMP-7 application and increased osteoblast number and osteoblast surface area in the ectopic bone without affecting osteoclast activity. At least during the experimental period, implantation of the dexamethasone pellet did not affect bone volume, trabecular thickness, osteoblast number, and osteoblast surface area of vertebrae and tibiae [55], which suggests that dexamethasone only affects osteoblast precursor or stem cells residing at sites with strong osteoblast induction such as those treated with BMPs. In our study, the scaffold-mediated delivery of dexamethasone and BMP-2 strongly promoted ectopic bone formation relative to BMP-2 alone, and this study is thus the first to show that local delivery of dexamethasone enhances the osteogenic effect of BMP-2 in vivo. Based on the results of our in vitro studies and in vivo studies, we speculate that both migrating muscle cells and circulating bone marrow-derived cells may have been exposed to the dexamethasone and BMP-2 in the scaffolds, resulting in their differentiation into osteoblasts to form ectopic bone. We used porous β-TCP blocks as a carrier for BMP-2 and dexamethasone and also as a scaffold for bone formation. It is well known that BMPs strongly adsorb onto calcium phosphate materials including β-TCP. Therefore, we consider that β-TCP is an appropriate carrier material for BMPs. However, we confirmed that dexamethasone does not adsorb onto β-TCP (data not shown) and may not be an appropriate carrier material for dexamethasone. Recently, the efficacy of dexamethasone-loaded CMCht/PAMAM dendrimer nanoparticles to enhance internalization of dexamethasone and subsequent osteoblastic differentiation of BMSCs was reported [56, 57]. Therefore, the use of dexamethasone loaded in such a carrier may further enhance the bone formation induced by BMP-2.

BMPs were approved for use in spine surgery by the US Food and Drug Administration late in 2002. Since then, BMP2 has been widely used not only for spinal fusion but also in other surgeries that require strong osteoinduction. However, side effects such as heterotopic bone formation [10–13], postoperative inflammation [12, 14–17], osteolysis and subsidence of implants [18–21], and cyst-like bone void formation [18] are of concern [22] and may result from excessive dosing of BMP-2. To overcome these negative aspects, many studies have attempted to develop BMP carriers to provide controlled release [58–62], whereas other studies have attempted to enhance the osteogenic inductivity of BMPs by combining them with other drugs or cytokines in vitro and in vivo [62–64]. However the findings of these trials have not yet been widely applied in the clinic. In the present study, addition of a small amount of dexamethasone to BMP-2 markedly augmented bone formation. Although dexamethasone has also been associated with complications such as osteoporosis and immunosuppression, the doses of dexamethasone used to augment bone formation in the present study were very low. Therefore, combined use of dexamethasone and BMPs may reduce the amount of BMPs required to achieve clinical efficacy, thus reducing both the cost of the procedure and also the side effect profile.

This study is not without limitations. Although we confirmed that the subpopulation selection effects of dexamethasone enhanced the differentiation capabilities of BMSCs and MuSCs, we have yet to specifically characterize the different cells in the heterogeneous cell population or to reveal the mechanism of subpopulation selection. Additionally, although the combination of dexamethasone and BMP-2 augmented bone formation both in vivo and in vitro in a rat model, it is well known that the response to BMPs is different among animal species. Therefore, for clinical applications, the quantities of dexamethasone and BMPs should be optimized.

## Conclusion

This is the first study to show that the combination of BMP-2 and dexamethasone augments the osteogenic differentiation of both BMSCs and MuSCs. We also demonstrated a strong effect of the combination of BMP-2 and dexamethasone on ectopic bone formation in vivo. These data suggest that dexamethasone could be used clinically to augment the effects of BMP-2 on bone formation. Further studies to elucidate the underlying mechanisms are required.

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## Author Contributions

Conceived and designed the experiments: MY T. Yoshii AO SS. Performed the experiments: MY T. Yamada TT TM WX MH HY SS. Analyzed the data: MY T. Yamada TT TM MH HY SS. Contributed reagents/materials/analysis tools: TU SS. Wrote the paper: MY SS.

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画像診断シリーズ　198

# ロコモシリーズ6　腰椎変性後側弯症

間世田優文　　上井　　浩　　徳橋　泰明
日本大学医学部整形外科学系整形外科学分野

## Ⅰ．定義・概念

加齢に伴って椎間板や椎間関節が変性して椎体を支える力が弱くなることや，骨粗鬆症による圧迫骨折も加わることによって，脊柱全体が後側方に弯曲してくる（後側弯）状態である．主な初期症状としては腰痛であるが，骨棘などの椎体変形や脊柱のねじれ（回旋変形）を伴ってくると，神経根や馬尾を圧迫するため，下肢のしびれ，疼痛や筋力低下が生じる場合も少なくない．また，後側弯が進行すると腰痛は悪化し，体幹のバランスも悪くなり，立位保持や歩行などの日常生活に支障を生じる．拘束性肺障害や逆流性食道炎などの合併症を認めることも少なくなく，様々な側面からQOLの低下につながっていくことが近年明らかになってきている[1,2]．

## Ⅱ．治　　療

保存療法としては非ステロイド性消炎鎮痛薬(NSAIDs)やビタミン剤（メチルメコバラミン），プロスタグランジン製剤(Prostagradin E1)などの薬物療法や，安静，体幹の屈曲運動を中心としたストレッチや筋力強化訓練，コルセットなどの装具療法，温熱療法などの物理療法，硬膜外ブロックや神経根ブロックなどの注射療法などがある．保存療法に反応しない症例に対しては手術を考慮するが，術式選択には議論がある．後側弯の程度によっては，除圧術のみでも対応可能な症例もあるが，脊柱のアラインメントを矯正するような侵襲の大きい手術が必要な症例もある．

成人の腰椎変性後側弯症の術式選択には多くの因子が関与する．近年，矢状面バランスには脊椎のみならず骨盤の形態も関与することが知られてきており，これらの指標も加味した計算式が提唱され，各種骨切り術を併用した矯正術が施行されている[3](Fig. 1)．

## Ⅲ．症例提示

症　例：77歳　女性
主　訴：腰痛，両下肢の痺れ，胸やけなどの消化器症状（胃食道逆流症）
既往歴：2007年　後側方固定術（L4-5）

2010年ころより徐々に腰が曲がってくるとともに症状が出現した．近医で保存的に加療したが改善しないため，2012年9月当院紹介受診されて，腰椎変性後側弯症の診断となった(Fig. 2)．

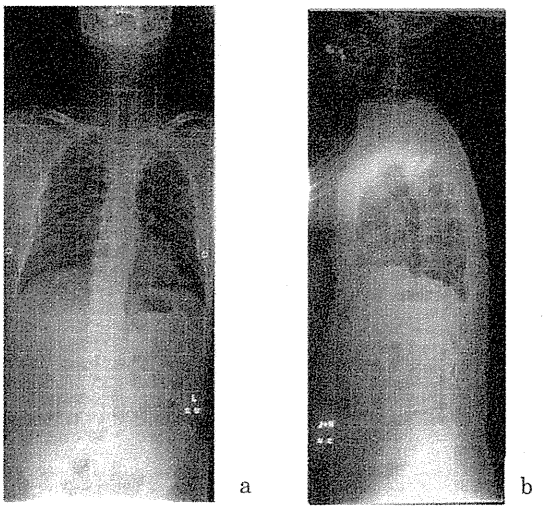

**Fig. 1**　Good global balance case of degenerative lumbar kyphoscoliosis
(a)　X-ray A-P view of total spine.
(b)　X-ray lateral view of total spine.

2012年10月手術（L3矯正骨切り術＋T8-腸骨後方固定術＋L2/3・3/4後方椎体間固定術）を行った．腰椎は術前13°の後弯から，術後は40°の前弯を形成し，53°の矯正を獲得できた．術後は腰痛が軽度残存するものの，体幹バランスの不良は改善されるとともに，両下肢の痺れと消化器症状は改善した(Fig. 3)．

## 文　　献

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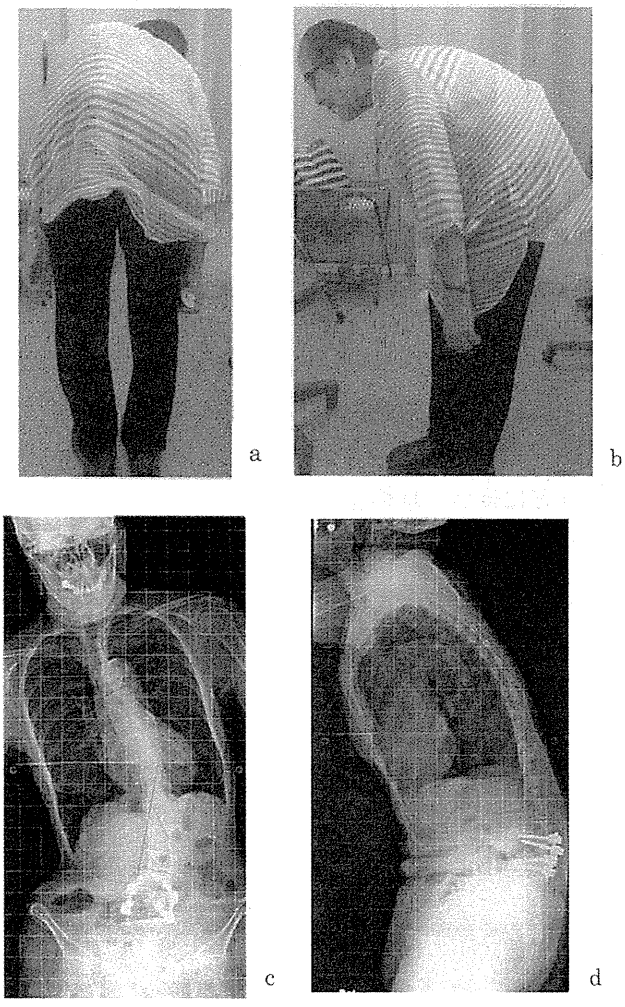

**Fig. 2** Preoperative image
(a) Posterior standing posture.
(b) Lateral standing posture.
(c) X-ray A-P view of total spine.
(d) X-ray lateral view of total spine.

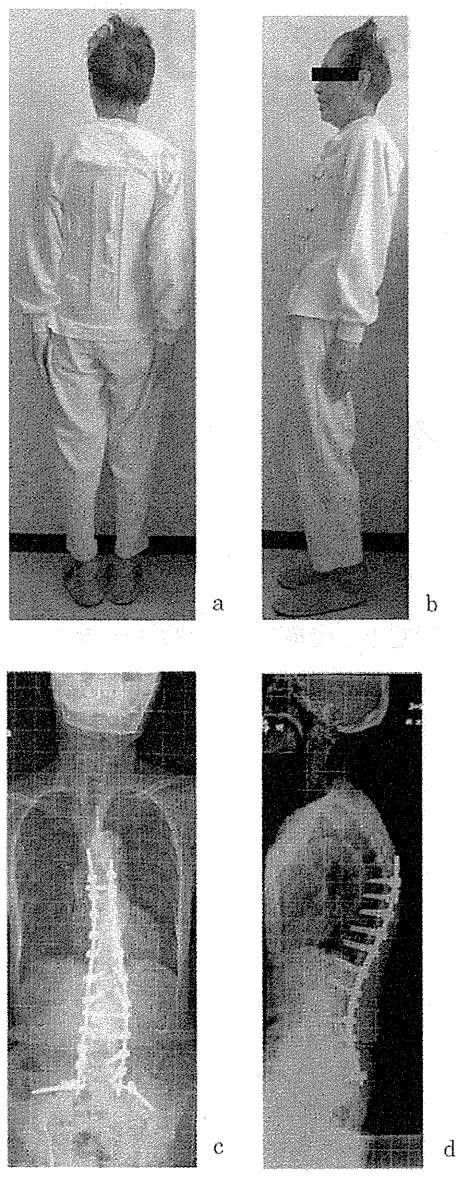

**Fig. 3** Postoperative image
(a) Anteroposterior standing posture.
(b) Lateral standing posture.
(c) X-ray A-P view of total spine.
(d) X-ray lateral view of total spine.

各論

# 骨折に対するギプス固定
# 椎体骨折 DVD 12

佐藤公昭
久留米大学医学部整形外科学准教授

永田見生
久留米大学学長

志波直人
久留米大学医学部整形外科学教授

## 骨折整復の基本手技

手術手技の向上や内固定材料の発達，装具療法の進歩・普及などに伴い，椎体骨折に対してギプスを用いた治療を行う機会は減少している。しかし，ギプス固定は疼痛の緩解，椎体変形の矯正および支持性の獲得が期待でき，骨折の保存療法や手術後の局所固定として簡便かつ有力な治療手段である。従って，ギプス固定の理論や手技について十分習熟し，取り扱う材料について理解を深めておく必要がある。

### ●椎体骨折整復の理論と分類

椎体骨折に対するギプス固定は，発生頻度の高い胸腰椎移行部の骨折治療を中心に発展してきた。基本的な治療原則は1920年代後半～1930年代にかけて確立されたものであるが，現在でも胸腰椎の圧迫骨折や破裂骨折などに対して十分な効果が得られる治療法である。ギプス固定の目的は，受傷部位の損傷拡大防止，局所の安静，整復位の保持，治癒促進などである。

脊椎不安定性の評価は統一されていないが，Holdsworthら[1]は，脊柱支持機構として脊柱後方靱帯（棘上靱帯，棘間靱帯，黄色靱帯）を重視し，脊椎損傷を表1のように分類した。

楔状骨折や破裂骨折あるいは伸展損傷では脊柱後方靱帯は温存されているので安定型損傷に分類できる。一方，脱臼および脱臼骨折あるいは剪断骨折は脊柱後方靱帯の損傷が高度なため不安定型損傷とした。不安定型損傷の場合は脊髄症状を伴うことが多く，構築学的な観点から脊椎損傷を安定性損傷と不安定損傷に大別したこの分類は，治療方針を決定するうえで参考になる。

表1　Holdsworthら[1]の脊椎損傷分類

| |
|---|
| ・安定型損傷（stable injuries） |
| a. 楔状骨折（simple wedge fractures） |
| b. 破裂骨折（burst fractures） |
| c. 伸展損傷（extension injuries） |
| ・不安定型損傷（unstable injuries） |
| a. 脱臼（dislocations） |
| b. 脱臼骨折（fracture dislocations） |
| c. 剪断骨折（shear fractures） |