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REGULAR ARTICLE

Forced expression of mouse progerin attenuates the osteoblast differentiation interrupting β-catenin signal pathway in vitro

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Abstract

Nuclear protein, lamin A, which is a component of inner membrane on nucleoplasm, plays a role in nuclear formation and cell differentiation. The expression of mutated lamin A, termed progerin, causes a rare genetic aging disorder, Hutchinson-Gilford progeria syndrome, which shows abnormal bone formation with the decrease in a number of osteoblasts and osteocytes. However, exact molecular mechanism how progerin exerts depressive effects on osteogenesis has not been fully understood. Here, we created mouse lamin A dC50 cDNA encoding progerin that lacks 50 amino acid residues at C-terminus, transfected it in mouse preosteoblast-like MC3T3-E1 cells, and examined the changes in osteoblast phenotype. When lamin A dC50-expressed cells were cultured with differentiation-inductive medium, alkaline phosphatase (ALP) activity and mRNA levels of major osteoblast markers, type I collagen (Col1), bone sialoprotein (BSP), dentine matrix protein 1 (DMP1), and Runx2 were significantly decreased, and no mineralized nodules were detected as seen in control cells expressing empty vector. In the culture with mineralization-inductive medium, mRNA levels of BSP, osteocalcin, DMP1, Runx2, and osterix were strongly decreased parallel with loss of mineralization in lamin A dC50-expressed cells, while mineralized nodules appear at 21 days in control cells. Furthermore, lamin A dC50 expression was depressed nuclear localization of β-catenin with the decrease of GSK-3β phosphorylation level. These results suggest that lamin A dC50 depresses osteoblast differentiation in both early and late stages, and it negatively regulates β-catenin activity interacting with GSK-3β in cytoplasm.

Keywords Lamin A · Progerin · β-Catenin signal · Osteoblast markers · Osteoblast differentiation

Introduction

Nuclear lamins of the intermediate filament make the nuclear membrane that provides the structural integrity of nucleus and the determination of cell differentiation (Stuurman et al. 1998). Two isoforms, lamin A and lamin C, which consists of 664 and 572 amino acid residues, respectively, are produced by alternatively splicing from 12 exons on LMNA gene (Fisher et al. 1986; McKeon et al. 1986). While lamin C is a direct product from exons 1 to 10 on LMNA gene, lamin A is translated from exons 1 to 12 and requires post-translational processing for the maturation, resulting in 18 amino acid residues at C-terminus are deleted (Corrigan et al. 2005; Mattout et al. 2006; Navarro et al. 2005). In contrast, aberrant lamin A causes a rare premature aging disorder, Hutchinson-Gilford progeria syndrome (HGPS), characterized by accelerated aging with bone abnormalities including severe osteoporosis and bone deformities (Hennekam 2006). In HGPS, a substitution of cytosine for thymine at nucleotide position 1824 of exon 11 of LMNA gene occurs irregular splicing, resulting in the

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expression of mutated lamin A, widely known as progerin, which completely lacks 50 amino acid residues at C-terminus (Corrigan et al. 2005; Eriksson et al. 2003; Goldman et al. 2004; Hennekam 2006).

Previous studies reported the features of bone abnormalities in HGPS. The mutant mice, which are disrupted from exons 8 to 11 on LMNA gene, showed severe osteopenia with low bone mass and bone deformities (Li et al. 2011). A mouse model of HGPS expressing a large amount of progerin leaded to severe bone abnormalities including spontaneous bone fractures, poorly mineralized bones, and widespread loss of osteocytes and osteoblasts (Mounkes et al. 2003; Schmidt et al. 2012; Yang et al. 2006). Furthermore, suppression of lamin A/C using small interfering (si) RNA showed the decrease in the expression levels of osteoblast makers, alkaline phosphatase (ALP), osteocalcin (OC), bone sialoprotein (BSP), and osterix (Osx), as well as the formation of mineralized nodules in human osteoblasts and bone marrow stromal cells (Akter et al. 2009; Rauner et al. 2009). However, exact molecular mechanisms how progerin inhibits osteogenesis are not well-understood.

It has been reported that β-catenin signal pathway is sufficient for osteoblast differentiation in mesenchymal stem cell (MSC) (Lin et al. 2016) or osteoprogenitors (Song et al. 2012; Sun et al. 2016). Activation of β-catenin depends on phosphorylation level in cytoplasm. Non-phosphorylated β-catenin is activated by dissociation from the complex with glycogen synthase kinase-3β (GSK-3β) and axin, moves into the nucleus, and binds to TCF/LEF motif on target genes. In contrast, phosphorylated β-catenin is captured by the complex with GSK-3β and axin, followed by ubiquitinated and resolved by proteasomes in cytoplasm (Monroe et al. 2012). Interestingly, overexpression of lamin A/C in MSCs strongly promoted osteoblast differentiation and activation of β-catenin for binding to TCF/LEF motif in nucleus (Bermeo et al. 2015), Furthermore, lamin A/C deficient mice showed low expression level of β-catenin and appearance of a large number of adipocytes in bone and muscle (Tong et al. 2011). These findings suggest that lamin A is a positive regulator in osteogenesis through the activation of β-catenin signal pathway.

Here, we examined the effect of mouse progerin, named lamin A dC50, which lacks 50 amino acid residues at C-terminus, on osteoblast differentiation of preosteoblast-like MC3T3-E1 cells. Lamin A dC50 was stably expressed in MC3T3-E1 cells, and the changes in osteoblast phenotype as well as the expression levels of β-catenin and GSK-3β were compared to control cells.

Materials and methods

Cell culture

A mouse preosteoblast cell line, MC3T3-E1 cells, was purchased from RIKEN Cell Bank (RCB1126, Tsukuba, Japan) and maintained in a growth medium consisted of α-minimum essential medium (Wako Chemical, Tokyo, Japan), 10% fetal calf serum, 10-U/ml penicillin, and 100-μg/ml streptomycin at 37 °C in the atmosphere of 5% CO_2. For each experiment, an appropriate number of cells were seeded on 24-well plates and cultured with growth medium until confluent. To initiate the differentiation of MC3T3-E1 cells, medium was replaced by growth medium containing 10^{-8}-M dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO), 10-mM β-glycerophosphate, and 50-μg/ml ascorbic acid (differentiation-inductive medium, D-medium), then the cells were cultured for 7, 14, and 21 days. Growth medium containing 10-ng/ml recombinant bone morphogenetic protein-2 (BMP-2, R&D System, Rochester, MN) and 10^{-8}-M Dex, 10-mM β-glycerophosphate and 50-μg/ml ascorbic acid (mineralization-inductive medium, M-medium) was also used to induce the terminal differentiation of MC3T3-E1 cells into mature osteoblasts capable for mineralization.

Construction of mouse lamin A dC50 cDNA

An approximate 1.8-kb mouse lamin A cDNA was used for construction of lamin dC50 (Tsukune et al. 2017). A short-length fragment with *Sph*I and *Not*I sites at 3′-end of mouse lamin A cDNA was amplified by PCR using oligonucleotide primers, P1: 5′-GCA TGC TCG CAC TAG CGG GCG TGT-3′ and P2: 5′-TTA CAT GAT GCT GCA GTT CTG GGA GCT CTG GGC TCC CGC TCC ACC GGC-3′. Amplified *Sph*I-*Not*I fragment were ligated into a long length fragment with *Xba*I-*Not*I sites of mouse lamin A cDNA (Fig. 1a) with the expression vector, pPyCAG-IP, whose EGFP sequence was deleted (Tsukune et al. 2017). This construct lacks 150-bp nucleotides that encode 50 amino acid residues containing a FACE1/Zmpste24 recognition site (Corrigan et al. 2005; Mattout et al. 2006; Navarro et al. 2005). A nucleotide sequence of lamin A dC50 was confirmed by single extension service (Sigma-Aldrich).

Transfection of lamin A dC50 into MC3T3-E1 cells

An expression vector encoding mouse lamin A dC50 was transfected into MC3T3-E1 cells using Lipofectamine LTX and plus reagent (Thermo Fisher Scientific, Waltham, MA) in the growth medium without antibiotics. After 5 h, medium was removed, fresh growth medium was added, and the cells were further cultured for 16 h. Positive clones expressing mouse lamin A dC50 were selected by adding 1-μg/ml puromycin (Sigma-Aldrich), and then subject to the experiments. As a control, pPyCAG-IP vector without sequences of lamin A dC50 and EGF was transfected into MC3T3-E1 cells.

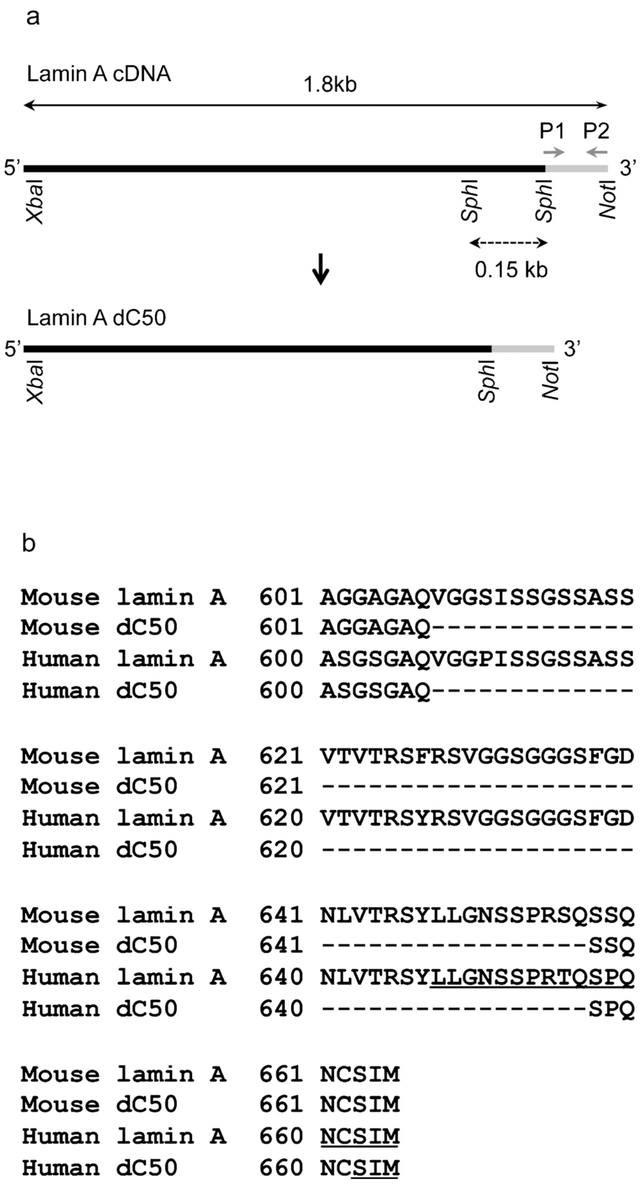


Fig. 1 Strategy for creating mouse lamin A dC50 and alignment of amino acid sequence at C-terminus of lamin A and lamin A dC50 in human and mouse. **a** Schematic representation of a 1.8-kb mouse lamin A cDNA (Tsukune et al. 2017). P1 and P2 indicated by arrows are sense and antisense primers for synthesizing a short fragment behind a deletion site composed of 150 nucleotides respectively. Long and short fragments encoding lamin A dC50 were ligated with expression vector, pPyCAG-IP as described in "Materials and methods." **b** C-terminal amino acid sequences of lamin A between mouse and human are well-conserved feature. In human, lamin A lacks 18 amino acid residues indicated by underline. In contrast, dot lines show deleted amino acid residues of lamin A dC50 (dC50) in human and mouse. In human dC50, substitution of cytosine for thymine at exon 11 causes irregular cleavage of 50 amino acid residues (Mattout et al. 2006). Similarly, created mouse cDNA encodes mouse dC50 that lacks 50 amino acid residues at the same position in human dC50

Standard RT-PCR and semi-quantitative RT-PCR

Total RNA was extracted from lamin A dC50-expressed MC3T3-E1 cells and control cells using RNeasy Mini Kit (Qiagen, Valencia, CA). The cDNA was synthesized from 1 μg of total RNA in 20 μl of reaction mixture containing 20-U reverse transcriptase (PrimeScript™ Reverse Transcriptase, Takara Bio, Shiga, Japan) at 42 °C for 30 min. One microliter of reaction mixture containing synthesized cDNA was subjected to standard PCR or real-time PCR.

Standard RT-PCR was performed in 20 μl of PCR mixture containing 1 μl of cDNA, 2.5-U *Taq* DNA polymerase, and oligonucleotide primers for lamin A (5′-GCA TGC TCG CAC TAG CGG GCG TGT-3′ and 5′-GCG GCC GC T TAC ATG ATG CTG CAG-3′), or β-actin (5′-ATC TAT GAG GGT TAC GCG CT-3′ and 5′-CTG TGG TGG TGA AGC TGT AG-3′). A reaction condition at 35 cycles is as follows: denaturing at 94 °C for 15 s, annealing and extension at 64 °C for 30 s, and final extension are 72 °C for 10 min. Ten microliters of PCR product was subjected to 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

The mRNA levels of ALP, type I collagen (Col1), BSP, OC, dentine matrix protein (DMP1), and β-actin were determined by real time PCR in 20 μl of reaction mixture containing cDNA and gene specific primer sets whose sequences are available in our previous report (Tsukune et al. 2017). In addition, primer sets for Runx2 (5′-CAC AAA TCC TCC CCA AGT GG-3′ and 5′-GGA GGG CCG TGG GTT CTG AG-3′) and Osx (5′-GGA GGT TTC ACT CCA TTC CA-3′ and 5′-TAG AAG GAG CAG GGG ACA GA-3′) were synthesized in this study. Real-time PCR was performed in Thermal Cycler using SYBR® Premix Ex *Taq*™ II (Takara Bio) according to the manufacturer's instruction. The mRNA level of each gene was analyzed using a software of Smart Cycler system (Cepheid, Sunnyvale, CA). The PCR condition at 30 cycles is as follows: denaturing at 95 °C for 5 s, annealing, and extension 60 °C for 20 s. All experiments were performed by triplicate and obtained data were normalized by expression level of β-actin, respectively.

Western blotting

The cells were lysed in a solution consisted of 50-mM Tris-HCl (pH 8.0), 150-mM sodium chloride, 0.5% (*w*/*w*) sodium deoxycholate, 0.1% (*w*/*v*) SDS, and 1% (*w*/*v*) NP-40. Cell lysates were centrifuged at 15,000 ×*g* for 15 min at 4 °C and supernatants were collected. Ten micrograms of protein was separated in a gradient SDS-PAGE gel (ATTO, Tokyo, Japan) and transferred to the polyvinylidene difluoride (PVDF) membrane. Subsequently, membrane was blocked with 5% skim milk in PBS at room temperature for 1 h. After washing with 0.1% Tween-20 in PBS, membrane was incubated with appropriately diluted first antibodies, polyclonal rabbit anti-lamin A/C antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse GFP antibody (Medical & Biological Laboratories, Nagoya, Japan), anti-phospho-GSK-3β (Ser9) antibody (Cell Signaling Technology, Denver, MA), anti-β-catenin antibody (BD PharMingen, San Jose, CA), or anti-active-β-catenin (Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C. After washing again, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was added as a secondary antibody at a dilution 1:5000 and incubated for 2 h at room temperature, and then protein bands were visualized by ECL detection kit (GE Healthcare, Buckinghamshire, England, UK) according to the manufacturer's instruction. Goat anti-mouse actin antibody (Santa Cruz Biotechnology) and HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) were also used for an internal control. The intensity of protein bands was converted into numerical value using ImageJ software (National Institute of Health, Bethesda, MD).

Histochemical analyses

The cells on 24-well plates were fixed with cacodylate buffer (pH 7.2) containing 10% formaldehyde for 30 min and then washed with PBS. ALP staining was performed using a solution dissolved with NBT/BCIP ready-to-use tablet (Roche Diagnostics GmbH, Penzberg, Germany). After washing with PBS, the cells were scanned using an Epson GT-X800 scanner (Seiko Epson, Tokyo, Japan).

For detection of mineralized nodules, the cells on 24-well plate were fixed with methanol for 10 min and stained with 1.0% Alizarin-Red S solution (Sigma-Aldrich) for 30 min. After washing with distilled water extensively, the plates were scanned. To measure the staining intensity, 5% formic acid was added in each well to extract the colored component released from mineralized nodules, and then absorbance was determined using a micro plate reader (SpectraMax 190, Molecular Devices, San Jose, CA) at O.D. 415 nm.

Reporter assay

Nuclear localization of β-catenin was examined using TOP/GFP plasmid that encodes TCF/LEF motif required for the binding of β-catenin in nucleus (Naito et al. 2012). TOP/GFP plasmid was transfected into the lamin A dC50-expressed MC3T3-E1 cells and control cells described above. After selection using 100-μg/ml geneticin (Sigma-Aldrich), GFP-, and TCF/LEF-expressed cells were further cultured with D-medium for 21 days. Subsequently, the cells were fixed with methanol for 10 min and then incubated with appropriately diluted anti-GFP antibody overnight at 4 °C. After washing with PBS (−), the cells were further incubated with an Alexa 488-conjugated anti-rabbit IgG antibody as a secondary antibody. GFP expression was detected under a fluorescence microscope (BZ-8100, KEYENCE, Osaka, Japan). Nuclear staining was performed using 1-μg/ml DAPI solution (Dojindo Laboratories, Kumamoto, Japan).

Recovery assay

Lamin A dC50-expressed MC3T3-E1 cells were cultured with D-medium in the presence or absence of 5-μM SB216763 (Sigma-Aldrich) or 5-μM deoxycholic acid (DCA, Sigma-Aldrich) for 21 days. Total RNA was extracted, and then real-time PCR was performed using primer sets for ALP and Col1.

Statistical analyses

Results indicated as mean ± SD of triplicate experiments and evaluated using the Student's *t* test or one-way Anova Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$ or < 0.001.

Results

Alignment of C-terminal amino acid sequences of lamin A and lamin A dC50 in human and mouse

In human, lamin A is composed of 664 amino acid residues that are translated from exons 1 to 12 on a LMNA gene, and post-translational processing at C-terminus occurs for mature lamin A, resulting in 18 amino acid residues, SIM, and LLGNSSPRSQSSQNC which are removed. As shown Fig. 1b, however, in human lamin A dC50, a substitution of cytosine for thymine on exon 11 causes aberrant cleavage of 50 amino acid residues from positions 607 to 656 near SPQNC sequence, although SIM sequence was moved by post-translational processing (Mattout et al. 2006). Similarly, mouse lamin A is encoded by exons 1 to 12 on LMNA gene, and 665 amino acid residues were translated (Nakajima and Abe 1995). Since C-terminal amino acid sequences of lamin A are well-conserved feature between human and mouse, we created mouse lamin AdC50 cDNA, which lacks 50 amino acid residues from 608 to 657, referring to deletion site of human dC50 (Fig. 1b). A created cDNA encoding mouse lamin A dC50 was inserted into expression plasmid, pPyCAG-IP, and stably transfected into MC3T3-E1 cells. RT-PCR detected mRNA expression of lamin A dC50 as a single band at a size of 556 bp below the band of intact lamin A at a size of 706 bp (Fig. 2a). Western blotting also showed a single band of lamin A dC50 above nearby that of lamin C (Fig. 2b), but undetectable expression of lamin A dC50 in control cells expressing empty vector.

Effect of lamin A dC50 on early differentiation in MC3T3-E1 cells

At first, confluent lamin A dC50-expressed MC3T3-E1 cells and control cells were cultured with D-medium and

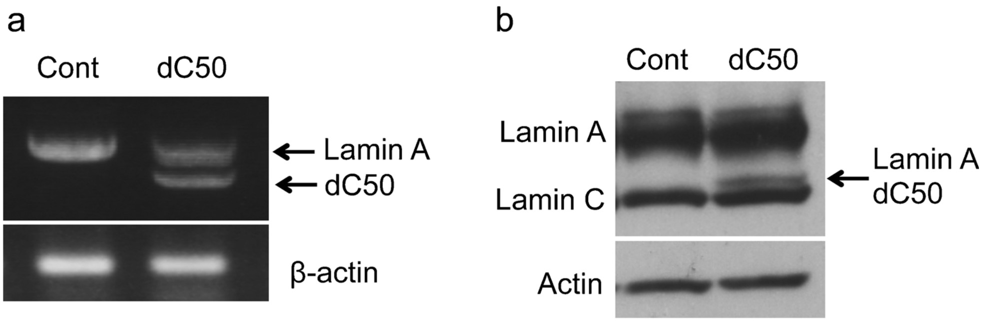


Fig. 2 Establishment of lamin A dC50-expressed MC3T3-E1 cells. **a** RNA was extracted from a positive clone selected as lamin A dC50-expressed MC3T3-E1 cells (dC50). RT-PCR was performed using specific primers for lamin A described in "Materials and methods." Upper band is intact lamin A and lower band is lamin A dC50. As a control, the cells expressing empty vector were used (Cont). The β-actin was also detected as an internal control. **b** Cell lysates from lamin A dC50-expressed MC3T3-E1 cells (dC50) were subjected to Western blotting using a polyclonal anti-lamin A antibody. Upper and lower bands indicate intact lamin A and lamin C, respectively. A single band above lamin C, which is indicated by arrow, shows the expression of lamin A dC50. As a control, the cells expressing empty vector (Cont) were used. The actin was also detected as an internal control

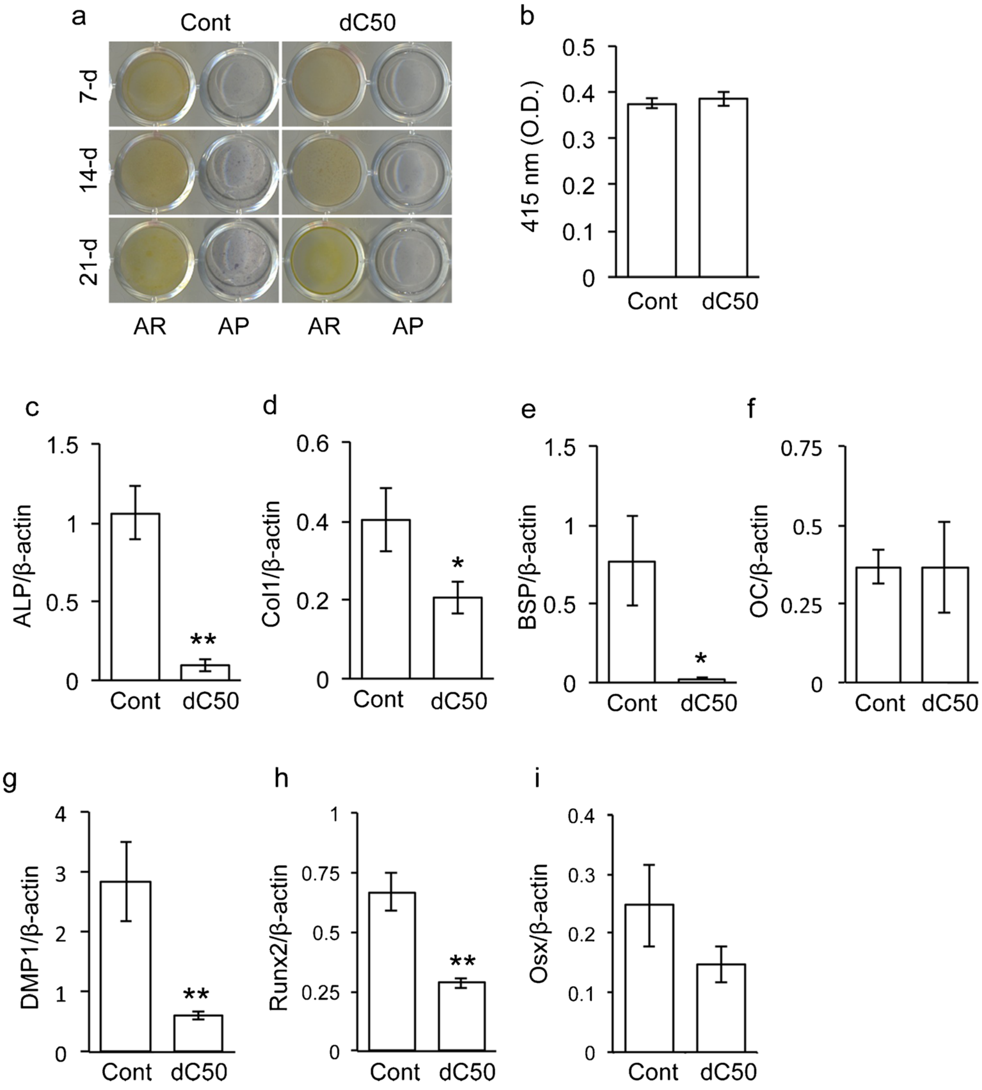


Fig. 3 Effect of lamin A dC50 on osteoblast differentiation in MC3T3-E1 cells cultured with D-medium. **a** Detection of ALP activity and mineralized nodules. Lamin A dC50-expressed MC3T3-E1 cells were cultured in D-medium for 7, 14, and 21 days (**d**). Staining for alizarin red S (AR) and ALP activity (AP) was performed. As a control, cells expressing empty vector (Cont) were used. **b** Intensity of alizarin red S staining in lamin A dC50-expressed cells (dC50) and control cells (Cont). After staining, colored component was extracted by formic acid, and then measured by O.D. 415 nm. **c**–**i** Semi-quantified PCR analysis for osteoblast markers in lamin A dC50-expressed cells (dC50) and control cells (Cont). The cells were cultured in D-medium for 21 days, and then real-time PCR was performed using specific primers for ALP (**c**), Col1 (**d**), BSP (**e**), OC (**f**), DMP1 (**g**), Runx2 (**h**), and Osx (**i**). Each expression level was normalized with that of β-actin. $*p < 0.05$ and $**p < 0.001$ compared with control

osteogenic activity was determined. The staining of ALP activity was weak, but mineralization nodules did not appear in both lamin A dC50-expressed cells and control cells at 7, 14, and 21 days of culture (Fig. 3a, b). In contrast, mRNA expression levels of osteoblast markers, Col1, ALP, BSP, OC, DMP1, Runx2, and Osx were clearly detected in control cells at 21 days (Fig. 3c–i). Furthermore, the decreased mRNA expression levels of ALP (23-fold decrease, $p < 0.001$, Fig. 3c), Col1 (2.1-fold decrease, $p < 0.05$, Fig. 3d), BSP (30-fold decrease, $p < 0.05$, Fig. 3e, DMP1 (4.7-fold decrease, $p < 0.001$, Fig. 3g), and Runx2 (2.3-fold decrease, $p < 0.001$, Fig. 3h) were shown in lamin A dC50-expressed cells compared to control cells, respectively. No significant change in mRNA expression levels of OC (Fig. 3f) and Osx (Fig. 3i) was not determined between lamin A dC50 expressed cells and control cells.

Effect of lamin A dC50 on mineralization in MC3T3-E1 cells

To define the effect of lamin A dC50 on mineralization activity of MC3T3-E1 cells, lamin A dC50-expressed cells were cultured in M-medium for 7, 14, and 21 days. ALP activity did not change between lamin A dC50-expressed cells and control cells at 7, 14, and 21 days of culture, but alizarin red S staining clearly showed the formation of mineralized nodules at 21 days in control cells (Fig. 4a). This mineralization activity was significantly decreased in lamin A dC50-expressed cells (3.5-fold decrease, $p < 0.001$, Fig. 4b) compared to control cells. Similarly, decreased mRNA levels of BSP (1.7-fold decreased, $p < 0.001$, Fig. 4e), OC (13-fold decrease, $p < 0.001$, Fig. 4f), DMP1 (1.7-fold decrease, $p < 0.001$, Fig. 4g), Runx2 (3.9-fold decrease, $p < 0.001$, Fig. 4h), and Osx (48-fold

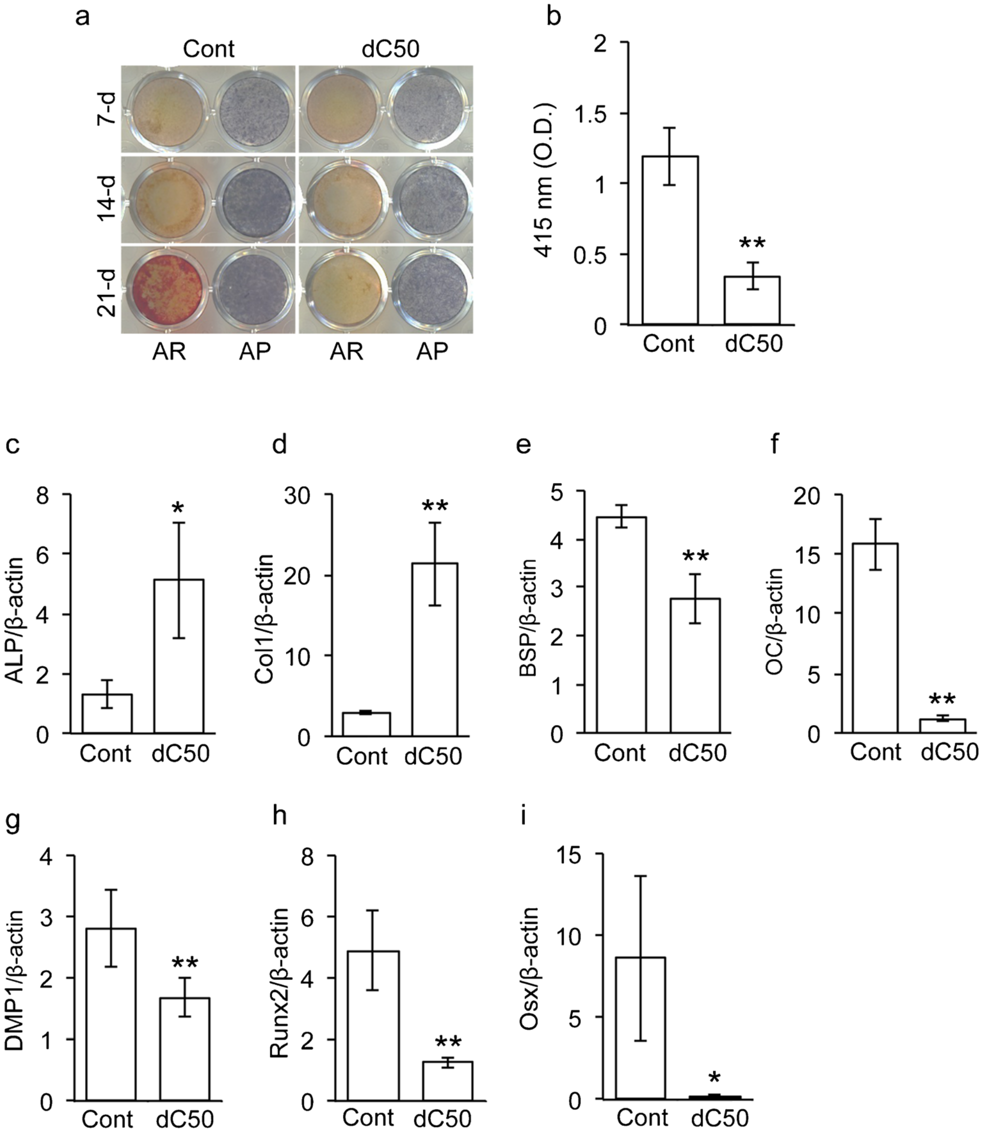


Fig. 4 Effect of lamin A dC50 on osteoblast differentiation in MC3T3-E1 cells cultured with M-medium. **a** Detection of mineralized nodules and ALP activity in lamin A dC50-expressed cells and control cells. Lamin A dC50-expressed MC3T3-E1 cells (dC50) were cultured in M-medium for 7, 14, and 21 days (**d**), and then the staining for alizarin red S (AR) and ALP activity (AP) was performed. As a control, the cells expressing empty vector (Cont) were used. **b** Intensity of alizarin red S staining in lamin A dC50-expressed cells (dC50) and control cells (Cont). After staining, colored component was extracted by formic acid, and then measured by O.D. 415 nm. $^{**}p < 0.001$ compared with control. **c**–**i** Semi-quantified PCR analysis for osteoblast markers in lamin A dC50-expressed cells (dC50) and control cells (Cont). The cells were cultured in M-medium for 21 day, and then real-time PCR were performed using specific primers for ALP (**c**), Col1 (**d**), BSP (**e**), OC (**f**), DMP1 (**g**), Runx2 (**h**), and Osx (**i**). Each expression level was normalized with that of β-actin. $^{*}p < 0.05$ and $^{**}p < 0.001$ compared with control

decreased, $p < 0.05$, Fig. 4i) were shown in lamin A dC50-expressed cells compared to control cells, respectively. In contrast, increased mRNA expression levels of ALP (3.9-fold increase, $p < 0.05$, Fig. 4c) and Col1 (7.1-fold increase, $p < 0.001$, Fig. 4d) were determined in lamin A dC50-expressed cells compared to control cells.

Effect of lamin A dC50 on β-catenin signal in MC3T3-E1 cells

To define the effect of lamin A dC50 on β-catenin signal pathway, reporter assay using a TOP/GFP plasmid was performed in lamin A dC50-expressed cells cultured with D-medium. Unlike in control cells that strongly express GFP (Fig. 5a, c), decreased GFP expression level was shown in nuclei of lamin A dC50-expressed cells (Fig. 5b, d). Western blotting also showed that GFP expression level was lower than that in lamin A dC50-expressed cells (3.4-fold decrease, $p < 0.05$, Fig. 6a, b) compared to control cells. Similarly, decreased expression levels were detected in active β-catenin (2.0-fold decrease, $p < 0.001$, Fig. 6a, c) and phosphorylated GSK-3β (1.6-fold decrease, $p < 0.05$, Fig. 6a, d) in lamin A dC50 expressed cells compared to control cells, respectively.

Effect of SB216763 and DCA on the β-catenin signal pathway in lamin A dC50-expressed MC3T3-E1 cells

To define whether if depressive effects of lamin A dC50 on osteoblast differentiation (Figs. 3c–e, g, h and 4a, b, e–i) are closely linked to the decreases in active β-catenin and phosphorylated GSK-3β, the recovery assay was performed using two different types of reagents, SB216763 and DCA. SB216763 acts as an inhibitor of GSK-3β to increase active β-catenin expression (Coghlan et al. 2000), while DCA is known as an activator of β-catenin in human colon cancer (Pai et al. 2004). Confluent lamin A dC50-expressed MC3T3-E1 cells (dC50) were cultured with D-medium in the presence or absence of DCA (DCA-treated dC50) or SB216763 (SB-treated dC50) for 21 days, and mRNA level of Col1 and ALP was determined. Notably, increased mRNA level Col1 (2.6-fold increase, $p < 0.05$, Fig. 7a) and ALP (2.5-fold increase, $p < 0.05$, Fig. 7b) was shown in DCA-treated dC50 compared to dC50, respectively. Furthermore, increased ALP mRNA level (2.3 fold increase, $p < 0.05$, Fig. 7b) was observed in SB-treated dC50. The ALP expression level in SB-treated dC50 was restored nearby that in control cells.

Discussion

This is a first report that mouse progerin, named lamin A dC50, depressed osteoblast differentiation at early and late stages in vitro. However, it is not fully proved weather if created mouse lamin A dC50 has negative functions in nuclear formation and cell development as seen in HGPS (Stuurman et al. 1998; Goldman et al. 2004; Mattout et al. 2006), because this product was made on the basis of deletion site of 50 amino acid residues in human progerin (Fig. 1b). Considering that C-terminal amino acid sequences of lamin A between human and mouse are well conserved, we believe that created lamin A dC50 is in accordance with mouse progerin that causes mouse progeria syndrome. In fact, RT-PCR and Western blot revealed that a clone expresses suitable signal as a single band between intact lamin A and C (Fig. 2a). In addition, negative effects of mouse lamin A dC50 on osteoblast differentiation and formation of mineralized nodule (Figs. 3a, b and 4a, b) resemble to the results in previous depressive studies using lamin A/C siRNA (Akter et al. 2009; Rauner et al. 2009). Therefore, it is suggested that lamin A dC50 will be useful for the research to elucidate the development of HGPS.

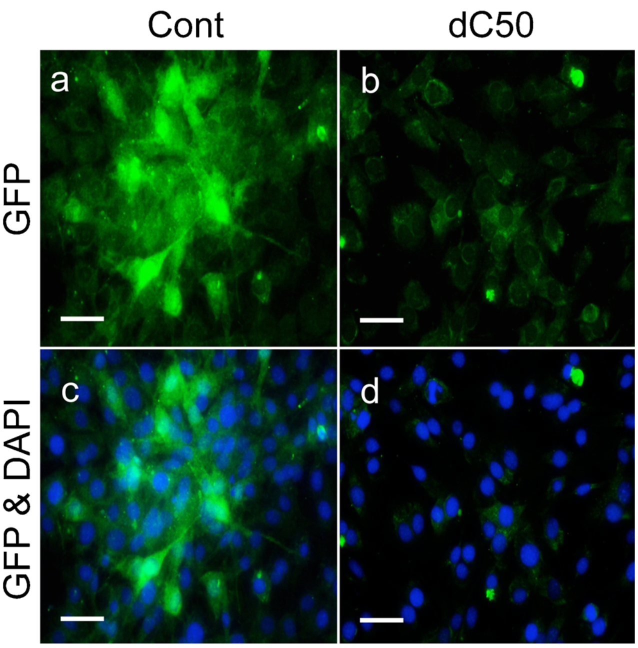


Fig. 5 The nuclear translocation of active β-catenin in lamin A dC50-expressed MC3T3-E1. **a**, **c** The control cells (Cont) expressing GFP and TCF/LEF were cultured with D-medium for 21 days. **b**, **d** Lamin A dC50-expressed cells (dC50), which was transfected with GFP and TCF/LEF motif, were also cultured with D-medium for 21 days. GFP expression was induced by the bind of β-catenin to TCF/LEF motif. Nuclear staining was performed using DAPI solution. Scar bar 100 μm

Effect of lamin A dC50 expression on early differentiation of MC3T3-E1 cells

Osteogenesis begins from the commitment of proliferative MSCs into the osteoblast lineages. MSC-derived osteoprogenitors become to preosteoblasts expressing Col1 and ALP, followed by mature osteoblasts that produce high level of osteoblast markers including OC, BSP, Runx2, and Osx, and form mineralized nodules (Nakashima and de

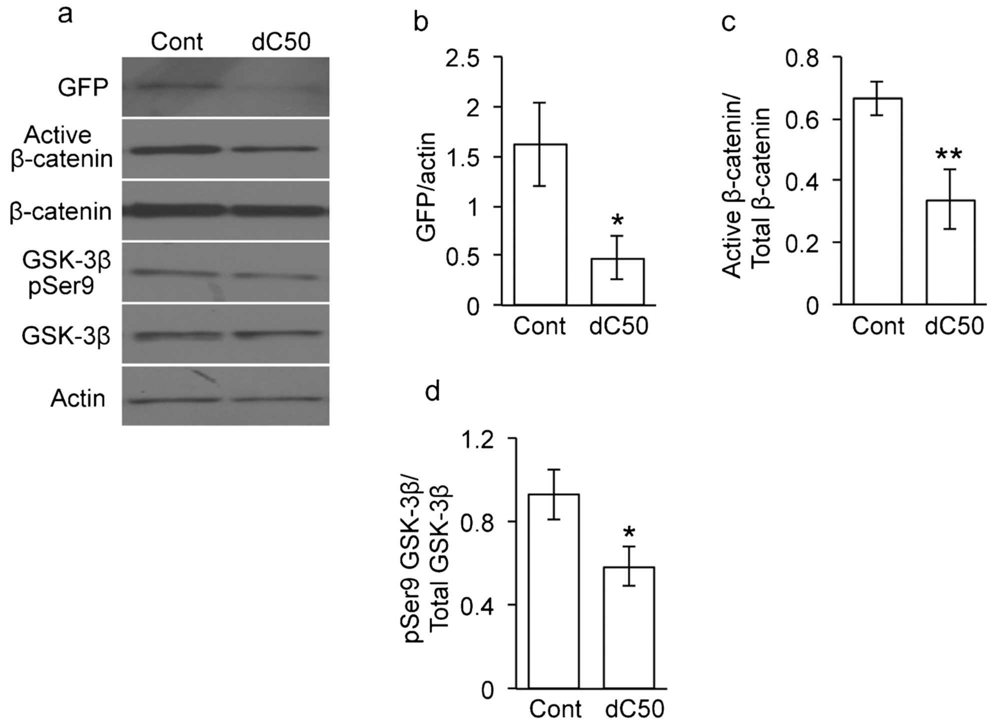
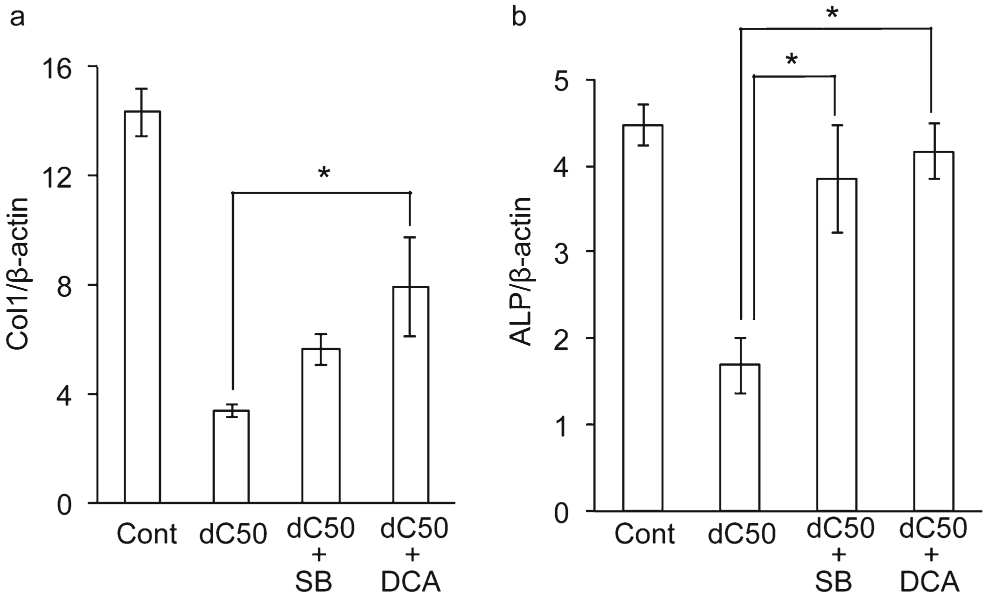


Fig. 6 Comparison of expression level of β-catein and GSK-3β in lamin A dC50-expressed cells and control cells. Lamin A dC50-expressed MC3T3-E1 cells (dC50) were transfected with a vector encoding the sequences of GFP and TCF/LEF, and then cultured with D-medium for 21 days. As a control (Cont), the cells expressing GFP and TCF/LEF were used. **a** Western blotting of β-catenin and GSK-3β. The expression level of GFP, total β-catenin (β-catenin), active β-catenin, phosphorylated GSK-3β, and total GSK-3β (GSK-3β) was detected using each specific antibody. Anti-actin antibody was also used as an internal control. **b**–**d** Expression levels of GFP (**b**), β-catenin (**c**), and GSK-3β (**d**) in lamin A dC50-expressed cells. Visualized bands of GFP, active β-catenin, and phosphorylated GSK-3β (Ser9) were scanned and converted into numerals after normalization by that of actin, total β-actin, and total GSK-3β, respectively. $*p < 0.05$ and $**p < 0.001$ compared with control

Crombrugghe 2003). Subsequently, mature osteoblasts undergo apoptosis or differentiate into bone lining osteocytes that express DMP1 strongly (Toyosawa et al. 2001; Komori 2010). A typical preosteoblast-like cell line, MC3T3-E1 cells, exhibits ALP activity and mRNA expressions of Col1, BSP and OC (Wang et al. 1999; Jadlowiec et al. 2004). They also express low levels of Runx2, Osx, and DMP1 before osteogenic stimulation (Narayanan et al. 2001; Jadlowiec et al. 2004). In this study, no mineralized nodules were shown in control MC3T3-E1 cells cultured with D-medium for 21 days

Fig. 7 Effect of SB216763 and DCA on Col1 and ALP expressions in lamin A dC50-expressed MC3T3-E1 cells. The effect of SB and DCA on mRNA expression of Col1 (**a**) and ALP (**b**) in lamin A dC50-expressed MC3T3-E1 cells. Lamin A dC50-expressed cells (dC50) were cultured with D-medium in the presence or absence of SB216763 (dC50 + SB) or DCA (dC50 + DCA) for 21 days. Real-time PCR was performed using specific primers for Col1 and ALP. As a control, the cells expressing empty vector (Cont) were used, and each expression level was normalized with that of β-actin. $*p < 0.001$ compared to dC50



(Fig. 3a, b), suggesting that they usually maintain a phenotype as preosteoblast lineage before beginning of terminal differentiation. In contrast, mRNA levels of ALP, Col1, BSP, DMP1, and Runx2 were significantly decreased in lamin A dC50-expressed cells (Fig. 3c–e, g, h), implying that lamin A dC50 prevents an early osteoblast differentiation of MC3T3-E1 cells. However, we cannot well explain that no significant change in mRNA levels of OC and Osx was shown between lamin A dC50-expressed cells and control cells (Fig. 3f, i). Probably, expression levels of OC and Osx in control cells are much lower, so that lamin A dC50 might be not able to interrupt their levels, although the cells were cultured with D-medium.

Effect of lamin A dC50 expression on terminal differentiation of MC3T3-E1 cells

On the other hand, mRNA levels of BSP, OC, DMP1, Runx2, and Osx as well as the formation of mineralized nodules were significantly decreased in lamin A dC50-expressed MC3T3-E1 cells cultured with M-medium at 21-day (Fig. 4e–i). Similarly, in osteoinductive MSCs or osteoblasts, lamin A/C siRNA decreased the expression levels of OC, BSP, and Osx with Runx2-binding activity to the promoter region of OC gene (Akter et al. 2009; Rauner et al. 2009). Furthermore, in lamin A/C-deficient mice, not only lower level of OC mRNA expression in long bone but also decreased bone mass in forelimbs and skull were observed (Schmidt et al. 2012; Yang et al. 2006). These findings suggest that lamin A dC50 also inhibits a terminal osteoblast differentiation of MC3T3-E1 cells, resulting in low level expressions of osteoblast markers and loss of mineralization.

Basically, BMP-2 activates the phosphorylation of BMP-signal molecules, such as Smad and mitogen-activated protein kinase, which are sufficient for osteoblast differentiation with expression of Runx2 and Osx (Chen et al. 2012). Therefore, it is considering that lamin A dC50 expression interrupts BMP-signal pathway for increasing the expression levels of osteoblast markers including BSP, OC, DMP1, Runx2, and Osx in MC3T3-E1 cells (Fig. 4e–i). However, mRNA levels of early osteoblast markers, ALP and Col1, were significantly increased in lamin A dC50-expressed cells cultured with M-medium (Fig. 4c, d). These results suggest that lamin A dC50 expression simultaneously activates other signal pathways required for Col1 and ALP inductions in MC3T3-E1 cells.

Effect of mutated lamin A expression on β-catenin signal in MC3T3-E1 cells

This study clearly indicated the interaction between lamin A dC50 and β-catenin in MC3T3-E1 cells. As shown in Fig. 5, high level of nuclear localization of β-catenin was detected as GFP-positive nuclei in control cells, but low level of β-catenin was shown in lamin A dC50-expressed MC3T3-E1 cells. Similarly, the expression levels of GFP (Fig. 6a, b) and active β-catenin (Fig. 6a, c) were significantly decreased. These results suggest that inhibitory effect of lamin A dC50 (Figs. 3c–e, g, h and 4a, b, e–i) is involved in the reduction of active β-catenin expression. Indeed, previous studies reported that suppression of lamin A/C decreased the expression level of β-catenin and its binding ability to TCF/LCF motif in human bone marrow-derived cells (Bermeo et al. 2015; Tong et al. 2011). Interestingly, the expression level of active β-catenin (Fig. 6a, c) and the phosphorylation level of GSK-3β (Fig. 6a, d) were simultaneously decreased. Dephosphorylated GSK-3β promotes the phosphorylation of β-catenin (inactive β-catenin) in a complex with GSK-3β and axin. Subsequently, phosphorylated β-catenin is ubiquitinated and resolved in proteasome (Monroe et al. 2012). Therefore, we are considering that depressive effect of lamin A dC50 depends on the interaction between β-catenin and GSK-3β in cytoplasm. Indeed, recovery assay showed that lamin A dC50-decreased mRNA levels of Col1 and ALP were restored in DCA- or SB216763-treated dC50, respectively (Fig. 7a, b). These results suggest that lamin A dC50-inactivated β-catenin signal leads to the decrease in ALP and Col1 expressions interacting with dephosphorylated GSK-3β in MC3T3-E1 cells at an early stage of osteoblast differentiation.

Present data strongly support that lamin A dC50 expression depresses the osteoblast differentiation through inactivation of β-catenin and dephosphorylated GSK-3β in MC3T3-E1 cells. However, direct interaction of lamin A dC50 with β-catenin and GSK-3β is not still clarified. Thus, further analyses to define the molecular mechanism between lamin A and β-catenin signal will be needed.

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