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ORIGINAL PAPER

A new murine osteoblastic cell line immortalized with the SV40 large T antigen

Miki Nakamura-Ota · Ryoji Hamanaka · Hiroyuki Yano · Sawako Adachi · Hideaki Sumiyoshi · Noritaka Matsuo · Hidekatsu Yoshioka

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Abstract The murine preosteoblastic cell line, MC3T3-E1, is widely used to study bone formation and differentiation in vitro. However, this cell line is unstable in culture. The current study was designed to establish a stable osteoblastic cell line. A mammalian expression vector carrying the SV 40 large T antigen was introduced into a primary culture of cells isolated from the calvaria of newborn mice. Among isolated cell lines, the MN16 cell line was selected for further characterization. The MN16 cell line was cultured for 28 days, and compared with the MC3T3-E1 cell line with or without induction. The expression of bone-related genes was examined using the real-time RT-PCR technique. Alizarin red and von Kossa staining were used to detect mineralization of nodules in the cultures. The cell line showed the characteristics of osteoblastic cells in term of gene expression patterns of various molecular markers and calcium deposition in the cell layer after induction. Furthermore, the MN16 cells showed strong adhesion to the basic domain of collagen, a result that is specific for bone-derived cells. The MN16 cell line was found to be stably differentiated into bone formation cells in vitro and should be useful for studying bone biology.

Keywords Bone formation · Cell culture · MC3T3-E1 cell · Osteoblastic cell line · SV40 large T antigen

Abbreviations

DMEM	Dulbecco modified Eagle's medium
α-MEM	Modified Eagle's medium
SDS	Sodium dodecyl sulfate

Introduction

Bone formation occurs through two different processes: intramembranous ossification and endochondral ossification. The former occurs in craniofacial bones and the lateral part of the clavicles, while the latter occurs in the long bones, vertebrae, the basal part of the skull and the medial part of the clavicles (Karsenty 2003). Osteoblasts, which play a central role in both types of ossification, produce a characteristic

M. Nakamura-Ota · H. Yano · S. Adachi · H. Sumiyoshi · N. Matsuo · H. Yoshioka (✉)
Department of Matrix Medicine, Faculty of Medicine, Oita University, 1-1 Hasama-machi, Yufu City, Oita 879-5593, Japan
e-mail: hidey@oita-u.ac.jp

M. Nakamura-Ota · R. Hamanaka
Department of Cell Biology, Faculty of Medicine, Oita University, 1-1 Hasama-machi, Yufu City, Oita 879-5593, Japan

H. Yano
Division of Radioisotope Research, Department of Research Support, Oita University, 1-1 Hasama-machi, Yufu City, Oita 879-5593, Japan

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extracellular matrix (ECM) and mineralize the bone matrix. In osteoblasts, collagen, alkaline phosphatase and osteopontin are expressed in the early stages of development, while bone sialoprotein (BSP) and osteocalcin are expressed in the late stages (Komori and Kishimoto 1998; Aubin and Heersche 2002; Choi et al. 1996).

MC3T3-E1 cells, which are derived from newborn mice calvaria, display a time-dependent and sequential expression of osteoblastic characteristics analogous to those observed in in vivo bone formation (Sudo et al. 1983). Therefore, MC3T3-E1 cells are widely used as an in vitro model of osteoblastic development. However, these cells are unstable due to instability of the mineralization in culture (Baba 2000).

The current study was designed to establish an osteoblastic cell line with functions such as stable production of bone substrate proteins and calcification in vitro. A mammalian expression vector carrying the SV 40 large T antigen, an immortalization gene (Gruenert et al. 1988), was introduced into a primary culture of cells isolated from the calvaria of newborn mice.

Materials and methods

Culture

ICR mice were used for the experiment. The animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Calvaria were removed aseptically from 5 mice (5-day-old) and cut into pieces. The pieces were digested in 5 ml of collagenase (collagenase A, Wako, Japan) solution (3 mg/ml in Dulbecco modified Eagle's medium (DMEM)) (sigma, St. Luis, MO, USA) at 37 °C for 30 min. After the solution was centrifuged at 1,000 rpm for 5 min, the supernatant was discarded. The pellet was suspended and digested in collagenase solution at 37 °C for 5 h again. After centrifugation, the pellet was suspended and cells were plated on three 100-mm dishes and cultured in DMEM containing antibiotics and 10 % fetal bovine serum (FBS) (Sanko Junyaku, Tokyo, Japan) at 37 °C in a 5 % CO_2.

Transfection with the SV40 gene and isolation of single clones

Primary cultured cells were grown to confluence in 100-mm dishes and transfected with 5 μg of linearized DNA of SV40 plasmids using the electroporation method (Gruenert et al. 1988). The transfected cells were plated in 60-mm dishes. For cloning, the cells were replated in a 100-mm dish with a density of approximately 100 cells/dish. The cells were obtained from independent colonies. Sixteen single clones were identified and characterized for their osteoblastic phenotype.

Induction of cell calcification

New cloned cells and MC3T3-E1cells (Riken cell bank, Ibaraki, Japan) were plated on 35-mm dishes at a density of 1×10^5 cell/dish and cultured in α-MEM (Modified Eagle's Medium) (Life technology Japan, Tokyo, Japan) and DMEM containing antibiotics and 10 % FBS at 37 °C in 5 % CO_2. The next day, after the cells were confirmed to have attached to the plate, the induction of cells was started. The medium was replaced with or without 10 mM of β-glycerophosphate and 50 μg/ml of L-ascorbic acid, replaced every third day (Bellows et al. 1986). The culture was continued for 14 and 28 days.

Alizarin red staining

After discarding the medium, the cells were washed with distilled water and fixed with 70 % ethanol. The fixed cells were washed with distilled water and subsequently stained with Alizarin red (Sigma-Aldorich, St. Louis, MO, USA) solution for 10 min (Dahl 1952). The remaining dye was washed out with distilled water.

von Kossa staining

After discarding the medium, the cells were washed with PBS and fixed with 10 % formalin. The fixed cells were washed with distilled water three times and dried. The cells were incubated with a 5 % silver nitrate solution and placed under ultraviolet light until the staining became dark. Subsequently, they were washed with distilled water three times and stained

with 5 % sodium thiosulfate for 1 min (Puchtler and Meloan 1978).

Quantitative analysis of the Alizarin red staining

A quantitative analysis of the staining for Alizarin red was performed. The microscopic areas of 10 mm^2 were chosen randomly (BZ-9000, Keyence, Osaka, Japan) and positive areas were analyzed using the BZ-II analyzing application software program (Keyence).

RT-PCR

Total RNA was extracted from culture cells using extraction kit (Invitrogen, CA, USA) according to the manufacture's instruction. Total RNA (5 μg) was reverse transcribed using MMLV reverse transcriptase kit (Invitrogen) with random hexamers at 37 °C for 1 h. The reaction sample was then heated at 95 °C for 5 min to stop reaction. After reverse transcription, PCR was performed in a 25 μl mixture containing 10 μl of reverse transcription the reaction product, using Go Taq Green Master Mix (Promega, WI, USA). Thirty-five cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min were performed for *SV40* and *GAPDH* genes. The gene-specific primers are listed in Table 1.

Real-time RT-PCR

Total RNA (1 μg) was reverse transcribed using reverse transcriptase (Toyobo, Tokyo, Japan) with random hexamers at 30 °C for 10 min and 42 °C for 50 min. The reaction sample was then heated at 94 °C for 10 min to stop reaction. For a quantitative analysis of the expression level of the mRNA, real-time PCR was performed using a LightCycler TaqMan Master (Roche, IN, USA). The thermal cycling conditions included 1 cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The relative mRNA expression level of *Osteocalcin*, *alkaline phosphatase*, *Col1a1*, *Col5a1*, *Sp7/Osterix* and *RANKL* genes was normalized against that of the *GAPDH* gene from the same RNA preparations using a comparative threshold cycle method (Page and Stromberg 2011). The primer sequences are listed in Table 1.

Table 1 Primers used for PCR procedures

1. For RT-PCR	
SV 40 (J02400)	
Sense	5′-ttaattgtaggctatcaacccg-3′
Antisense	5′-ggttaataaggaatatttgatgtatagtgc-3′
GAPDH (BC145812)	
Sense	5′-agaggtgctgcccagaa-3′
Antisense	5′-gtggggagacagaagggaacaga-3′
2. For real-time RT-PCR	
Osteocalcin (NM007541)	
Sense	5′-agactccggcgctacctt-3′
Antisense	5′-ctcgtcacaagcagggttaag-3′
Alkaline phosphatase (NM007431.2)	
Sense	5′-cggatcctgaccaaaaacc-3′
Antisense	5′-tcatgatgtccgtggtcaat-3′
Col1a1 (NM007742.3)	
Sense	5′-catgttcagctttgtggacct-3′
Antisense	5′-gcagctgacttcagggatgt-3′
Col5a1 (NM 015734.2)	
Sense	5′-ctacatccgtgccctggt-3′
Antisense	5′-ccagcaccgtcttctggtag-3′
Sp7/Osterx (NM 130458.3)	
Sense	5′-agagatctgagctgggtagagg-3′
Antisense	5′-aagagagagcctggcaagagg-3′
RANKL (AF053713.1)	
Sense	5′-tgaagacacactacctgactccg-3′
Antisense	5′-ccacaatgtgttgcagttcc-3′
GAPDH (BC145812)	
Sense	5′-caatgaatacggctacagcaac-3′
Antisense	5′-ttactccttggaggccatgt-3′

The accession number is shown in the parentheses

Southern blotting

Genomic DNA was isolated from the culture cells. The cells were scraped, lysed and digested in a solution containing proteinase K (500 μg/ml) at 50 °C for 2 h. After the reaction was completed, the DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100 % ethanol. DNA was washed with 70 % ethanol, dried, solved in TE buffer and stored at 4 °C until use.

The DNA was digested with restriction endonuclease, subjected to 0.7 % agarose gel electrophoresis and transferred to nylon (Nihon Poll Ltd, Tokyo, Japan) using the methods of Southern blotting. The filters were hybridized in a solution containing 50 %

formamide, 5× SSC, 5× Denhardt's solution (0.1 % polyvinylpyrrolidone, 0.1 % Ficoll, and 0.1 % bovine serum albumin), 1 % sodium dodecyl sulfate (SDS), 5 % dextran sulfate and 100 μg/ml of salmon sperm DNA at 65 °C with the ^{32}P-labeled probe, using the random-primer method. After hybridization was completed, the filters were washed with 0.5× SSC and 0.1 % SDS at 65 °C four times and the bands were analyzed with the imaging analyzer BAS2000 (Fuji film, Tokyo, Japan).

Cell adhesion assay

The recombinant protein of the pro-α3(V)239/371 collagen fragment was generated as previously described (Puchtler and Meloan 1978). The cell adhesion assay was performed as also previously described (Puchtler and Meloan 1978). In brief, the proteins were put in 24-well plate (2 μg/cm^2) at room temperature for 2 h. The solution was removed and the well was blocked with 1 % BSA for more than 1 h. The blocking solution was removed, and the wells were washed with PBS. The cells were plated in each well with a concentration with 1 × 10^5, and incubate at 37 °C for 1 h. After washing, attached cells were fixed with 4 % paraformaldehyde in PBS and stained with 0.5 % crystal violate in 10 % ethanol for 30 min. After washing, cells were solubilized with 1 % SDS, the absorbance was measured at a wavelength of 595 nm.

Statistical analysis

The values represent the mean ± SD of multiple independent tests and Student's *t* test was used to evaluate the statistical differences between groups, and a $p < 0.05$ was considered to be significant. Each experiment was performed at least three times and individual samples were run in triplicate.

Results

Sixteen murine osteoblastic cell lines were established using cells isolated from the calvaria of 5-day-old mice. Among these lines, the MN 16 cell line was selected due to its high expression of osteocalcin and type I collagen and strong staining with Alizarin red

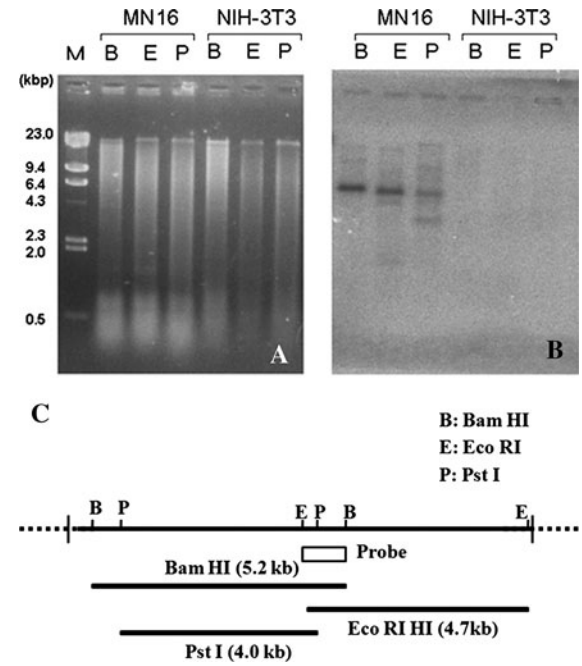

Fig. 1 Southern blot analysis. Ten μg of DNA extracted from MN16 and NIH-3T3 cells were digested with *Bam HI* (*B*), *Eco RI* (*E*) and *Pst I* (*P*), respectively, run on 0.7 % agarose gel (**a**), and blotted on the filter and hybridized with the ^{32}P-labeled probe (**b**). *M* DNA size marker. The restriction map of the construct DNA, which is integrated in a chromosome, is shown in (**c**). *Dotted lines* of both sides are the portions of chromosome DNA

14 days after differentiation. Therefore, MN 16 cells were used for further experiments.

A Southern blot analysis was performed to confirm the integration of the plasmid DNA containing the SV40 large T antigen gene in the MN 16 cells. The DNA was digested with restriction endonuclease, Bam HI, Eco RI and Pst I. The expected fragments, 5. 2, 4.7 and 4.0 kb in size digested with Bam HI, Eco RI and Pst I, respectively, were seen using the isotope-labeled probe of the Bam HI/Eco RI fragment derived from the SV40 large T antigen gene (Fig. 1a–c). Transcripts derived from the SV40 large T antigen were also detected during the culture of 28 days with or without differentiation (data not shown).

The expressions of bone-related genes, including osteocalcin, alkaline phosphatase, type I and V collagens, Runx2, Sp7/osterix and RANKL, were examined using the real-time RT-PCR technique to characterize the MN16 cells (Fig. 2). The alkaline phosphatase level strongly increased after the

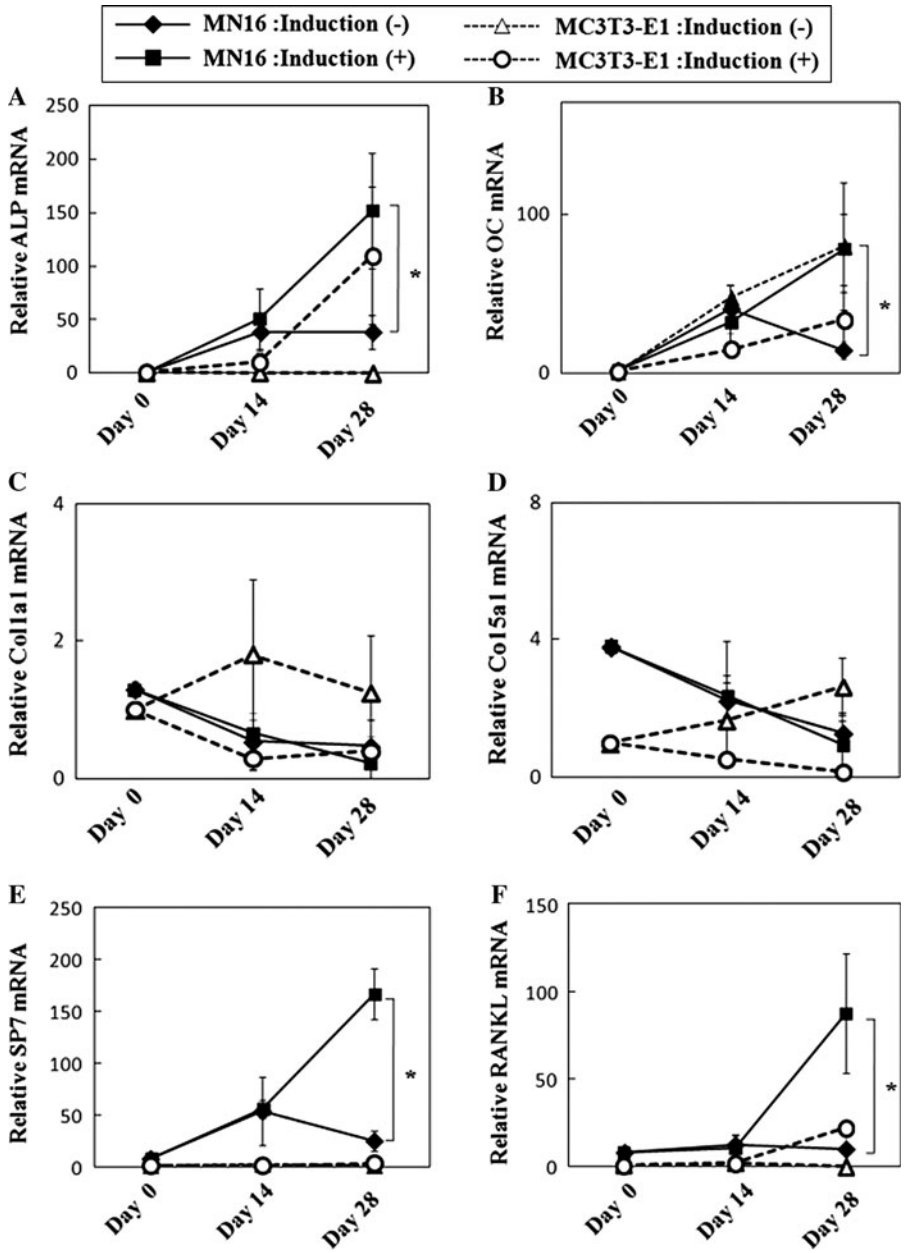

Fig. 2 Real time RT-PCR analysis. The expressions of alkaline phosphatase (**a**), osteocalcin (**b**), type α1(I) collagen (**c**), type α1(V) collagen (**d**), Sp7/Osterix (**e**) and RANKL (**f**) 14 and 28 days after differentiation of MN16 and MC3T3-E1 cells. GAPDH was used to normalize each expression level. The value of the MC3T3-E1 cells before differentiation was regarded as 1.0. The data are expressed as the mean ± SD of at least three independent experiments. The asterisks indicate statically significant results in the MN16 cells with or without induction. $*p < 0.05$. The cells were cultured in α-MEM

induction (Fig. 2a). Osteocalcin was strongly up-regulated in the MN16 cells from day 14 to day 28 after differentiation (Fig. 2b). Two collagen genes, α1 chains of type I and V collagen, were moderately expressed at the beginning of the culture, then their levels gradually decreased during differentiation (Fig. 2c, d). A transcription factor, Sp7/osterix, was up-regulated during differentiation (Fig. 2e). The expression of RANKL strongly increased on day 28 (Fig. 2f). These experiments were also performed in parallel using MC3T3-E1 cells as controls. In general, the values of the MC3T3-E1 cells were lower than those of the MN16 cells. Reverse expression patterns were also seen in these two cell lines. For example, the expression of type I collagen was higher under the undifferentiated condition of the MC3T3-E1 cells (Fig. 2c).

Alizarin red and von Kossa staining were used to detect mineralization of nodules in the cultures (Fig. 3). The MN16 cells were strongly stained with

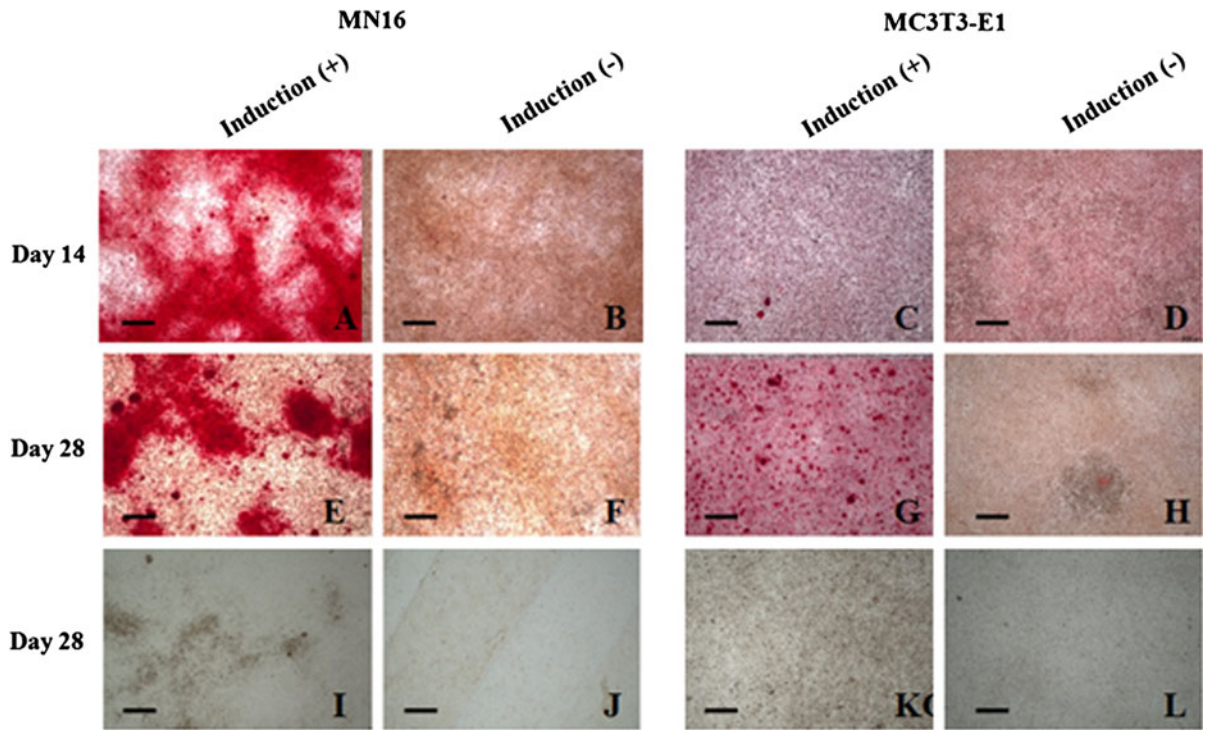

Fig. 3 Alizarin red (**a**–**h**) and von Kossa (**i**–**l**) staining. The MN16 (**a**, **b**, **e**, **f**, **i**, **j**) and MC3T3-E1 cells (**c**, **d**, **g**, **h**, **k**, **l**) were cultured for 14 days (**a**–**d**) and 28 days (**e**–**l**) with (**a**, **c**, **e**, **g**, **i**, **k**) or without (**b**, **d**, **f**, **h**, **j**, **l**) induction. The histological images used are representative of all replicates. The cells were cultured in α-MEM. *Bars* 300 μm

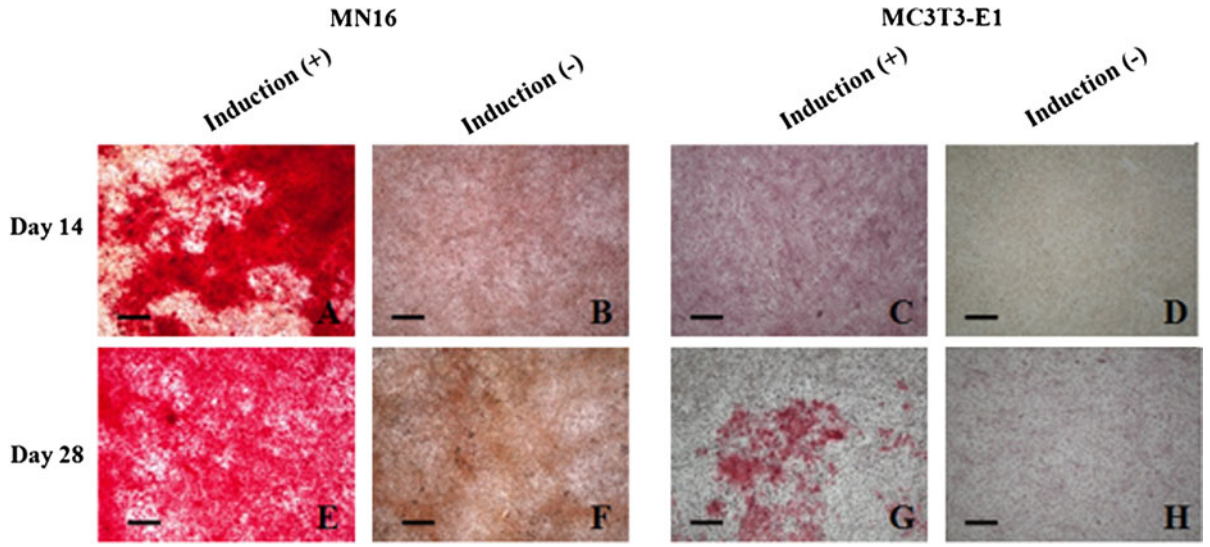

Fig. 4 Alizarin red (**a**–**h**) staining. The MN16 (**a**, **b**, **e**, **f**) and MC3T3-E1 cells (**c**, **d**, **g**, **h**) were cultured for 14 days (**a**–**d**) and 28 days (**e**–**h**) with (**a**, **c**, **e**, **g**) or without (**b**, **d**, **f**, **h**) induction. The histological images used are representative of all replicates. The cells were cultured in DMEM. *Bars* 300 μm

Table 2 Quantitative analysis of the Alizarin red staining

	14 days	28 days
MN16		
α-MEM	1.00 ± 0.21	0.70 ± 0.17
DMEM	1.07 ± 0.38	0.94 ± 0.08
MC3T3-E1		
α-MEM	n.d.	0.57 ± 0.04*
DMEM	n.d.	0.27 ± 0.13*

The value of the MN16 cells on 14 days after differentiation was regarded to be 1.00. The data are expressed as the mean ± SD of three independent experiments. The asterisks indicate statically significant results in the MC3T3-E1 cells compared to that of MN16 cells in α-MEM medium on 14 days. * $p < 0.05$. *n.d.* not detectable. The signals were not detected without differentiation in any experiments (data not shown)

Alizarin red on day 14 and 28 after differentiation (Fig. 3, panels a, e), whereas the MC3T3-E1 cells used in this experiment were barely stained on day 14 and weakly stained on day 28 (Fig. 3, panels c, g). Similarly, the MN16 cells were positive for von Kossa staining on day 28 (Fig. 3, panel i). The cells were also cultured in DMEM instead of α-MEM. As shown in Fig. 4, the staining patterns using Alizarin red in DMEM were similar to those in α-MEM. The qualitative analysis of Alizarin red staining by microscopy confirmed that the mineralization on day 14 and 28 was more prominent in the MN16 cells compared to the of MC3T3-E1 cells (Table 2).

A previous study showed that the basic segment derived from pro-α3(V) collagen preferentially adheres to rat ROS 17/2.8 sarcoma cells but not to rat RCS chondrosarcoma cells (Yamaguchi et al. 2005). In this experiment, it strongly adhered to MN16 and MC3T3-E1 cells and barely to rat RCS chondrosarcoma cells (Fig. 5).

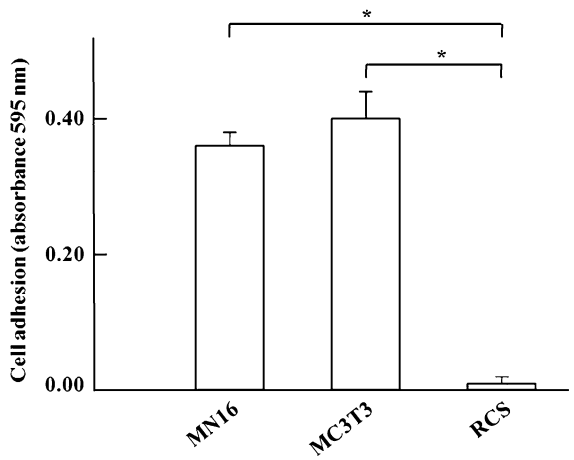

Fig. 5 Cell adhesion assay. MN16, MC3T3-E1 and RCS cells were plated on dishes coated with the recombinant protein of pro-α3(V) collagen polypeptide. The values shown are the mean ± SD of three experiments. The *asterisks* indicate statically significant results between the cells. *$p < 0.05$. The cells were cultured in α-MEM

Discussion

Various primary cells and cell lines have been employed for studying osteoblast cell biology. The primary cultures show the characteristic features of osteoblasts in vitro. However, they can undergo limited passages and sometimes show a heterogeneous phenotype due to the tissue source of tissues and/or method of preparation. To overcome these disadvantages, immortalized cell lines have been developed. The most commonly used cell lines are SaOs-2, MG-63 and MC3T3-E1 (Czekanska et al. 2012). The SaOs-2 cell line was derived from a human sarcoma, and does not show the whole range of osteoblast phenotype changes. The MG-63 cell line derived from a human juxtacortical osteosarcoma arrests in the pre-osteoblastic state. The MC3T3-E1, preosteoblastic murine cell line, was originally established from newborn calvaria based on high alkaline activity in the resting state (Sudo et al. 1983). However, this cell line sometimes appears to be unstable due to a loss of ability to achieve mineral deposition (Baba 2000). Therefore, this study aimed to establish a cell line with osteoblastic characteristics that is stably expressed in vitro. The MN16 cell line was selected on the basis of a high expression of osteocalcin and type I collagen and positive staining with Alizarin red 14 days after differentiation. The cell line shows the characteristics of osteoblastic cells in terms of gene expression of various molecular markers for osteoblast and calcium deposition in the cell layer after induction. However, alkaline phosphatase, an early marker, and osteocalcin, a late marker showed a similar expression pattern in the experiment. Since the MN16 cell line was selected as a clone with a high expression of osteocalcin after induction, the parental cell from which the MN16 cell line was derived might has had characteristics of preosteoblasts before transfection. Therefore,

osteocalcin might have been expressed rather earlier in the MN16 cell line.

For osteoblastic cells, α-MEM is the standard medium to promote the cell growth and survival (Hinoi et al. 2002). However, The MN16 cells could be cultured in DMEM and thus demonstrated similar characteristics to those cells (Fig. 4). Each gene expression examined was higher in the MN16 cell line than in the MC3T3-E1 cell line overall. The MN16 cell line shows a stable and reproducible expression pattern, and is superior to the MC3T3-E1 cell line in term of its osteoblastic characteristics. In conclusion, the data described above showed that the MN16 cell line exhibits immature osteoblastic characteristics and can be differentiated to osteoblast in which calcium deposition occur in the cell layer similar to bone matrix in vivo. Therefore, this cell line should be an excellent tool for studying bone biology.

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Conflict of interest The authors report no conflicts of interest.

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