THESIS

Controlled Differentiation of Stem Cells by Tetracycline-Controlled Transcriptional Activation of Amelogenin

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Introduction

Amelogenin is secreted by ameloblasts during tooth development, and it comprises more than 90 % of developing extracellular enamel matrix proteins. Amelogenin is the primary component of Emdogain® (1, 2), which is used clinically to regenerate periodontal defects (3-8). Although amelogenin has traditionally been considered as an enamel protein, the biological activity of amelogenin in the process of cell differentiation has recently become widely recognized. A previous study reported that specific amelogenin gene splice products induced *in vivo* mineralization accompanied by the expression of bone matrix proteins, such as *bone sialoprotein* (*BSP*) (9). Viswanathan *et al.* demonstrated that recombinant amelogenin slightly enhanced *BSP* expression in cementoblasts at the lowest dose; while dramatically decreased *BSP* expression at the highest dose. They also revealed that amelogenin null mice displayed a dramatic reduction in the expression of *BSP* in cementoblasts and surrounding osteoblasts, indicating that amelogenin is a potential regulator of cementum-associated genes (10).

At a low level of amelogenin expression has been reported in non-dental cell types, including stem cells, bone cells, brain and other soft tissues (11), suggesting additional functions of amelogenin such as a role in signal transduction in these cells.

Histological observations showed that amelogenin expresses in normal tissues of alveolar bone at a low level, and increases its expression at sites of high bone activity and remodeling (12). In addition, several studies suggest that amelogenin has a unique function to modulate osteogenic differentiation of stem cells. In mouse embryonic stem (ES) cells, exogenous leucine-rich amelogenin peptide was demonstrated to rescue partially amelogenin-null phenotype, and significantly increased the expression of *BSP* and *Osterix* during osteogenic differentiation (13). In human bone marrow-derived mesenchymal stem cells (MSCs), full-length amelogenin enhances the mRNA level of *alkaline phosphatase* (*ALP*), *type I collagen* and *BSP* as well as extracellular matrix (ECM) mineralization (14). Hu *et al.* (15) performed genome-wide expression profiling of human MSCs which were transduced with lentivirus encoding *amelogenin*, and showed up-regulation of osteogenic differentiation genes, such as *bone morphogenetic protein-2* (*Bmp2*), *Bmp6*, *Osteopontin* and *vascular endothelial growth factor C* (*VEGFC*). However, the mechanisms by which amelogenin contribute to the osteogenesis of MSCs, especially, the effects of amelogenin on the mineralization has not been fully understood.

Mammalian tooth development is largely dependent on sequential and reciprocal epithelial-mesenchymal interactions (16-18). During tooth development, amelogenin is

known to be involved in organizing of enamel rods (19), which plays a crucial role in the biomineralization and structural organization of enamel (20, 21). In this process, amelogenins have been identified to be nanospheres, self-assembled gydrophobic molecules, which regulate the growth, shape and size of hydroxyapatite crystals (20, 22-25). After that, amelogenins are eventually degraded by the metalloprotease enamelysin and the serine protease KLK4 (26), then finally replaced by mineral ions of calcium and phosphorus, leading to fully mineralized mature enamel (27-29). Although amelogenins are documented to be potential epithelial-mesenchymal signaling molecules during tooth development (30), far less is known about the role and mechanism of its action.

On the other hand, *Bmp4* has been shown as one of the key signaling molecules in tooth development (odontogenesis) (31, 32). Expression of *Bmp4* shifts from dental epithelium to dental mesenchyme during early tooth development that is associated with a shift of the tooth developmental potential from the dental epithelium to the mesenchyme (33). Especially, Bmp4 acts as a key *Msx1*-dependent mesenchymal odontogenic signal for driving tooth morphogenesis (34-38). In addition, mesenchymal *Bmp4* has been demonstrated to be in charge of the induction and maintenance of specific signaling molecules, leading to the final tooth development (39, 40). Therefore,

Bmp4 could play an important role of the tooth-inductive capability.

Induced pluripotent stem cells (iPSCs) have the ability to differentiate into all derivatives of the three primary germ layers including dental epithelial and mesenchymal cells (41, 42). Because iPSCs have great potential as tools for regenerative medicine/dentistry, these cells are expected to be used in the future regenerative dental therapy for the missing jaw bones and the lost teeth (43). However, only a little data, so far, is available about tooth development by the epithelial-mesenchymal interaction using iPSCs. Therefore, controlled expression of key molecules in the odontogenesis, such as *amelogenin* and *Bmp4*, in iPSCs may provide a new strategy to generate a bioengineered tooth.

To investigate the direct effect of *amelogenin* expression on differentiation of stem cells, forced expression of *amelogenin* by the lentiviral transduction is one of the most powerful and cost-effective methods (44). In this system, the viral genome integrates into host chromosomes, and the inserted gene is maintained in the cells over the multiple passages to express the target gene. In addition, lentiviral vectors can infect efficiently non-dividing cells as well as dividing cells in contrast to conventional retroviral vectors (45). These properties of the lentiviral transduction facilitate permanent and efficient expression of the transgene in stem cells even after their

proliferation and differentiation. However, the undifferentiated state of stem cells often lose their intrinsic stemness property after the viral transduction because the method leads uncontrollable expression of the transgene, which often precludes a generation of clonal cell culture in an undifferentiated state. This problem makes it difficult to perform reproducible experiments of stem cells using over-expression systems.

Tetracycline (Tet)-dependent transcriptional regulatory system is one of the best studied transduction systems with proven efficacy of controllable gene expression where transcription is reversibly turned on or off in the presence or absence of a tetracycline (Tet) derivative (doxycycline: Dox) (46, 47). This system (T-REx system) is based on the binding of Dox to the Tet-repressor (TetR) and de-repression of the promoter controlling expression of the gene of interest. It has been reported that the Tet-dependent system has been successfully used to control gene expression with the lentiviral transduction systems *in vitro* (48-53). Therefore, Tet-inducible gene transcription using the lentiviral transduction system could be a powerful method to investigate and control the functions of amelogenin in stem cells.

In this study, I focused on the Tet-dependent lentiviral transcriptional regulatory system to control forced expression of exogenous *amelogenin* (*Amelx*: *amelogenin, X-linked*) and *Bmp4* genes in stem cells. The objectives of this study were first to

establish a Tet-regulated system for *Amelx* expression in MSCs and iPSCs, and second to investigate effects of exogenous expression of *Amelx* on osteogenesis of MSCs, and third to explore a possibility to induce a bioengineered tooth by interaction of *Amelx* expressing iPSCs and *Bmp4* expressing iPSCs.

Materials and methods

Ethics Statement

All animal experiments in this study strictly followed a protocol approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry (approval number: 25-001-0).

1. Establishment of a Tet-regulation system in MSCs and iPSCs

1) Cell culture

Immortalized clonal mouse bone marrow-derived MSCs (54) were maintained in the growth medium (control medium) consisting of the modified Eagle's medium-alpha media (α-MEM) (Cat# 21444-05, Nacalai tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS) (Lot# JBS-8683, Japan Bio Serum, Hiroshima, Japan) and Antibiotic-Antimycotic (100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B) (Cat# 15240-062, Life technologies, Carlsbad, CA). During the process of osteogenesis, osteogenic induction medium consisting of the α-MEM supplemented with 15% FBS (Cat# 490082, Life technologies), Antibiotic-Antimycotic (100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B), 0.1 μM dexamethasone (Cat# D2915, Sigma, St. Louis, MO); 10 mM

β-glycerophoshate (Cat# G9422, Sigma) and 50 μM ascorbate-2-phosphate (Cat# A8960-5G, Sigma) was used.

293FT cells (Cat# R700-07, Life technologies), which were cultured for producing lentivirus, was maintained in the Dulbecco's modified Eagle's medium (DMEM 4.5 g/L glucose without sodium pyruvate; Cat# 08459-35, Nacalai tesque) with 10% FBS (Lot# JBS-8683, Japan Bio Serum, Hiroshima, Japan), 0.1 mM MEM non-essential amino acids (NEAA) (Cat# 11140-050, Life technologies), 6 mM L-glutamine (Cat# 25030-081, Life technologies), 1 mM MEM sodium pyruvate (Cat# S8636, Sigma), 1% penicillin/streptomycin (50 units/mL penicillin and 50 μg/mL streptomycin, Cat# 15140-122, Life technologies).

Mouse gingiva-derived iPSCs (55) were used in this study and the iPSCs were maintained in the ES cell medium consisting of the DMEM, 15% FBS (Lot# 10437, Life technologies), 2 mM L-glutamine, 10^{-4} M NEAA, 10^{-4} M 2-mercaptoethanol (Cat# 21985-023, Life technologies) and 0.5% of penicillin/streptomycin. SNLP76.7-4 feeder cells were supplied by Dr. Allan Bradley of the Sanger Institute (London, UK) and cultured in DMEM supplemented with 7% FBS (Lot# JBS-8683, Japan Bio Serum), 1% of 2 mM L-glutamine and 0.5% of penicillin/streptomycin.

2) Production of lentiviral vectors carrying *Amelx* or *Bmp4* gene

pENTR Directional TOPO cloning kit, pLenti6.3/Tet operator (TO)/V5 expression vector, pLenti6.3/V5-GW/*EmGFP* (*Emerald green fluorescent protein*) expression vector and Virapower™ Packaging Mix were used in this study. The pCMV-SPORT6.1 plasmid vector containing a full-length cDNA of mouse *amelogenin, X-linked* (*Amelx*: GenBANK：BC059090.1) was purchased from Open Biosystems (Thermo Scientific). An open reading frame of the *Amelx* cDNA (660 bp) was PCR-amplified (Forward primer CAC CAT GGG GAC CTG GAT TTT GTT; Reverse primer TCA TTT TTC TGT TGT GCT TTC C), and was cloned into the pENTR™/D-TOPO vector using pENTR Directional TOPO cloning kit (Life technologies) to obtain the entry vector (pENTR™/D-TOPO/*Amelx*) for the Gateway® cloning system (Life technologies). Using this entry vector, expression vector of *Amelx* [pLenti6.3/TO/V5/*Amelx*] was constructed by the LR recombination reaction of the Gateway® cloning system.

The Gateway® entry vector (pENTR221) containing a full-length human *Bmp4* cDNA (GenBANK：EU176183.1) was purchased from Open Biosystems and the expression vector (pLenti6.3/TO/V5/*Bmp4*) was constructed using the Gateway® cloning system. The recombination of the *Bmp4* cDNA in the pLenti6.3/TO/V5/*Bmp4*

was confirmed by a DNA sequence using the M13 (-21) or M13 reverse primer [M13 (-21) forward primer TGTAAAACGACGGCCAGT; M13 reverse primer CAGGAAACAGCTATGAC]. Expression of *Amelx* or *Bmp4* gene in the pLenti6.3/TO/V5/*Amelx* or pLenti6.3/TO/V5/*Bmp4* was examined by reverse transcription-polymerase chain reaction (RT-PCR) analysis using a primer set shown in **Table 1**.

293FT cells were cultured in 6 cm-dishes to produce the lentivirus by the pLenti3.3/*TetR* (Life technologies), pLenti6.3/TO/V5/*Amelx* and pLenti6.3/TO/V5/*Bmp4* expression vectors. PLenti6.3/V5-GW/*EmGFP* expression vector (Life technologies) was used as a control vector to examine the transduction efficiency.

Three microgram of Virapower^{TM} Packaging Mix (Life technologies), 1 μg of the expression vector and 12 μl of lipofectamin 2000 (Cat# 11668-019, Life technologies) were mixed in 1 ml OPTI-MEM I (Cat# 31985-062, Life technologies). After 25 minute-incubation, the mixture was added to 293FT cells. Forty eight hours later, the virus-containing supernatant was collected using a 10 ml-disposable syringe, and then filtrated with a 0.45 μm-pore size cellulose acetate filter.

3) Establishment of Tet-controlled *Amelx* expression system in MSCs

MSCs were cultured in 6 cm-dishes in the growth medium. When the cells reached 80% confluence, medium was replaced with the lentiviral stock solution of pLenti3.3/*TetR*, and the cells were cultured overnight at 37℃, 5% CO_2. Then, the lentivirus-containing medium was replaced with fresh growth medium. After 3 days, the cells were treated with geneticin (500 μg/ml) (Cat# 10131-035, Life technologies). After 5 days, survived cell colonies were picked up to generate MSC clones (MSCs-*TetR*) which strongly express *TetR* gene. Expression of *TetR* in MSCs-*TetR* was examined by RT-PCR analysis using a primer set shown in **Table 1**.

MSCs-*TetR* were seeded at a cell density of 3×10^5 cells in a 6 cm-dish in the growth medium, and were incubated overnight at 37℃, 5% CO_2. Then, medium was replaced with the viral stock solution supplemented with 4 μg/ml polybrene (Cat# 17736-44, Nacalai tesque). After 24 hours, cells were washed once with PBS and were cultured in fresh growth medium. After 5 days, cells were treated with 10 μg/ml blasticidin S (Cat# KK-400, Funakoshi, Tokyo, Japan) to select the colony of MSCs-*TetR* which express *Amelx* (MSCs-*TetR*/*Amelx*). Tet-dependent expression of *Amelx* gene in MSCs-*TetR*/*Amelx* was evaluated by RT-PCR (a primer set is shown in **Table 1**) and Western blotting analyses in the presence or absence of Dox (2 μg/mL).

4) Generation of blasticidin S resistance SNL feeder cells

SNL cells (feeder cells of iPSCs) were lentivirally infected with the pLenti6.3/V5-GW/*EmGFP* expression vector which contains the blasticidin S resistance gene (*Bla*) sequence. The transduced SNL cells (SNL-*Bla*) were selected by blasticidin S (10 μg/mL). SNL-*Bla* feeder cells were cultured on 10 cm-dishes, and when cells reached 90% confluence, cells were inactivated by treatment with mitomycin-C (Cat# 20898-21, Nacalai tesque) for 2.5 hours. Cells were then seeded in 6-well plates at a cell density of 2×10^5 cells/well, and were cultivated overnight before use.

5) Establishment of Tet-controlled *Amelx*/*Bmp4* expression system in iPSCs

iPSCs were cultured at a cell density of 2×10^5 cells/well in a 6-well plate (containing inactivated SNL feeder cells which originally express neomycin resistance gene) in the ES cell medium. When iPSCs reached 50% confluent, medium was replaced with lentiviral stock solution of pLenti3.3/*TetR*, and cells were incubated overnight at 37℃, 5% CO_2. The viral stock solution of pLenti6.3/V5-GW/*EmGFP* was also used to monitor the transduction efficiency. After the overnight incubation, the lentivirus-containing medium was replaced with fresh ES cell medium. After 3 days,

cells were treated with geneticin (500 μg/ml). After 5 days, survived colonies, which were expected to express the *TetR* gene (iPSCs-*TetR*), were picked up. To increase the expression level of *TetR* gene in the selected iPSCs-*TetR* clones, the transduction method was repeated 7 times on the same iPSCs-*TetR* clones using 10 mg/mL of a geneticin treatment. Expression of TetR gene in the iPSCs-*TetR* was evaluated by RT-PCR (using a primer set shown in **Table 1**) and Western blotting analysis.

Next, iPSCs-*TetR* were seeded at a cell density of 2×10^5 cells/well in a 6-well plate (containing inactivated SNL-*Bla* feeder cells) in the ES cell medium. When the cells reached 50% confluent, medium was replaced with lentiviral stock solution of pLenti6.3/TO/V5/*Amelx* or pLenti6.3/TO/V5/*Bmp4* supplemented with 4 μg/ml polybrene, and cells were incubated overnight at 37℃, 5% CO_2. After 24 hours, cells were washed once with PBS, and were cultured in fresh ES cell medium with 400 μg/ml blasticidin S. After 5 days, survived colonies, which were expected to express the *Amelx* (iPSCs-*TetR*/*Amelx*) or *Bmp4* (iPSCs-*TetR*/*Bmp4*) gene, were picked up. To increase the expression level of *Amelx* or *Bmp4* gene in the selected iPSCs-*TetR*/*Amelx* or iPSCs-*TetR*/*Bmp4* clones, the transduction method was repeated 3 or 2 times, respectively. Tet-dependent expression of *Amelx* gene and protein in iPSCs-*TetR*/*Amelx* were evaluated by RT-PCR (a primer set is shown in **Table 1**) and Western blotting

analysis, respectively, in the presence or absence of Dox (2 μg/mL). Tet-dependent expression of *Bmp4* in iPSCs-*TetR*/*Bmp4* was evaluated by RT-PCR (a primer set is shown in **Table 1**) in the presence or absence of Dox (2 μg/mL).

2. Tet-controlled transcriptional activation of *Amelx* during MSC osteogenesis

1) Effects of forced expression of *Amelx* on osteogenesis of MSCs-*TetR*/*Amelx*

MSCs-*TetR*/*Amelx* were cultured in the control or osteogenic induction medium in the presence or absence of Dox (2 μg/mL) for 7-35 days. Tet-dependent expression of *Amelx* and osteogenic marker genes [*Osterix*, *Osteocalcin*, *Osteopontin*, *BSP*, *type I collagen*] were evaluated by semi-quantitative RT-PCR (primer sets are shown in **Table 1**) and quantitative real-time RT-PCR analyses (primer sets are shown in **Table 2**). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control.

ALP activity was analyzed by ALP staining (56). The cells in 24-well plates were washed by PBS once and fixed in 10% buffered formalin phosphate for ALP assay. Cells were stained by incubating with 1.8 mM fast red TR (Cat# F8764-1 Sigma) and 0.9 mM naphthol AS-MX phosphate (Cat# N4875, Sigma) in 120 mM Tris buffer (pH 8.4) for 30 minutes at 37 °C.

The mineralized nodule formation was evaluated by von Kossa staining (57). After washing with PBS and following fixation with 10 % buffered formalin phosphate, the cells in 24-well plates were soaked in 5% AgNO3 (Cat# S6506, Sigma) and were exposed to UV light for 20 minutes. After that, the cells were washed by distilled water and were treated with 5% $Na_2S_2O_3$ (Cat# 197-03605, Wako) for 5 minutes.

The calcium deposition was evaluated by Alizarin Red S staining (58). After washing with PBS and following fixation with 10 % buffered formalin phosphate, the cells in 24-well plates were soaked in 40 mM Alizarin Red S (Cat# A5533-25G, Sigma) for 20 minutes with a gentle shaking. After washing by distilled water, the samples were scanned (GT-9800F, Epson, Tokyo) to obtain digital images of the stained samples. Next, 400 μl of 10% acetic acid (Cat# 01-0270, Sigma) were added to the sample and the samples were incubated at room temperature for 30 minutes to perform a quantitative analysis (59). Briefly, the stained sample was collected using a cell-scraper and was transferred into a 1.5 ml tube. After a heating treatment at 85℃ for 10 minutes and a following cooling treatment on ice for 5 minutes, the sample was centrifuged at 20,000 ×g for 15 minutes. The colored supernatant (250 μl) was collected into a new 1.5 ml tube and 100 μl of 10% ammonia (Cat# 016-03146, Wako) was added to each tube. Optical density of the supernatant sample was measured at 405 nm.

3. Tet-controlled transcriptional activation of *Amelx*/*Bmp4* in iPSCs

1) Teratoma formation and histological analysis

To investigate whether forced expression of *Amelx* in iPSCs-*TetR*/*Amelx* shows lineage-specific differentiation, *in vivo* differentiation assay (teratoma formation assay) was performed. iPSCs-*TetR*/*Amelx* were cultured on the SNL-*Bla* feeder cells on 6-well plates in the presence or absence of Dox (0.2 μg/mL). When the cells reached 70% confluence，the cells were transferred to low-attachment 10 cm-dishes to form embryonic bodies (EBs) (55), and were cultured in the mouse ES medium under a floating condition in the presence or absence of Dox. Medium was changed every two days. After 21 days of the floating culture, EBs of iPSCs-*TetR*/*Amelx* were collected by centrifugation at 300 rpm for 2 minutes.

Twenty microliters of the cell suspension ($0.2\text{-}0.5\times10^6$ cells/testis) were injected into the medulla testes of eight-week-old immunodeficient mouse (C.B-17 SCID; Clea Japan, Tokyo, Japan) using a Hamilton syringe. The mice were thereafter housed with free access to water and food under specific pathogen-free conditions. After 10 weeks, the teratomas were excised and subjected to histological analysis. Specimens were embedded in paraffin, and sectioned at 3 μm for hematoxylin and eosin (H&E) staining.

2) Co-culture of *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs to induce odontogenic differentiation

To investigate whether interaction of *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs triggers odontogenic differentiation, EBs of iPSCs-*TetR*/*Amelx* and iPSCs-*TetR*/*Bmp4* were co-cultured in mouse ES medium in the presence or absence of Dox (0.2 μg/mL) for 21 days. The expression of ameloblast-lineage marker, *Amelx* and odontoblast-lineage marker, *dentin matrix acidic phosphoprotein 1* (*DMP1*), was determined by the quantitative real-time RT-PCR analysis (primer sets are shown in **Table 2**).

4. RT-PCR analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). After DNase I treatment (Ambion, Austin, TX), cDNA was synthesized from 1 μg of total RNA using Super Script III reverse transcriptase (Life technologies). The cDNA target was amplified by PCR using Taq DNA polymerase (Promega, Madison, WI) following the manufacturer's recommendations. The primer pairs used are given in **Table 1**. PCR products were subjected to 1.5 % agarose gel electrophoresis with

ethidium bromide staining and visualized under ultraviolet light illumination. The expression of *GAPDH* mRNA was used as an internal control.

For quantitative real-time PCR analysis, SYBR Green assay and TaqMan assay were performed using Thunderbird SYBR qPCRMix (Cat# TMMFS-001, Toyobo, Osaka) and TaqMan Gene Expression PCR Master Mix (Life Technologies, Applied Biosystems, Foster City, USA), respectively. The primer pairs used are shown in **Table 2**. A PCR machine used in this study was Applied Biosystems 7300 realtime PCR system (Applied Biosystems, Foster City, USA). Expression of *GAPDH* mRNA was used as an internal control. Quantitative analysis of target gene expression was performed using the $\Delta\Delta$Ct method (60).

5. Western blotting analysis

When the cells reached 90% confluence, cells were washed once by cold PBS (-) (Cat# 14249-95, Nacalai tesque), and collected into 50 ml conical tubes. After centrifugation at 300 rpm for 5 minutes, cell pellets were lysed with RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, and 0.1% SDS] supplemented with protease inhibitor cocktail (Nacalai tesque). Proteins from the cell lysates were separated on 10% SDS-polyacrylamide gel

electrophoresis, and transferred to a polyvinylidine difluoride membrane (Millipore). The blot was blocked with TBST (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween-20) containing 5% skim milk, and then incubated with primary monoclonal antibody of TetR (1:1000; clone 9G9, Cat# 631131, Takara, Japan), amelogenin (1:1000; clone F11, Cat# sc-365284, Santa Cruz Biotechnology, Santa Cruz, CA), or GAPDH (1:10000, clone 6C5, Cat# MAB374, Millipore) at 4 °C overnight. After washing with TBST, the membrane was incubated with anti-mouse IgG HRP-linked antibody (1:3000, Cat# 7076, Cell Signaling) for 1 hour at room temperature. Signals were detected with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and the FPM 100 imaging system (Fujifilm, Tokyo, Japan).

Results

1. Establishment of a Tet-controlled *Amelx* expression system in MSCs

1) Establishment of MSCs-*TetR*/*Amelx*

Ten days after the transduction of MSCs with the pLenti3.3/*TetR* expression vector, few colonies were observed in the culture containing 500 μg/mL geneticin (**Fig. 1A**). The colonies were picked up and clonal culture of MSCs (MSCs-*TetR*) was established. RT-PCR result showed that the MSCs-*TetR* strongly expressed *TetR* mRNA (**Fig. 1B**).

To generate Tet-controlled *Amelx* expression system in MSCs (MSCs-*TetR*/*Amelx*), supernatant with lentivirus coding *Amelx* was first prepared using 293FT cells (**Fig. 1D**). The transfection efficiency was monitored by evaluation of *GFP* expression. The efficiency of transfection into 293FT cells was typically > 90%, as indicated by *GFP* expression (**Fig. 1C**).

Seven days after the transduction of MSCs-*TetR* with the pLenti6.3/TO/V5/*Amelx* expression vector, survived cells in the culture containing 10 μg/mL Blasticidin S were collected, and used as MSCs-*TetR*/*Amelx* (**Fig. 1E**).

2) Inducible expression of *Amelx* in MSCs-*TetR*/*Amelx* by Dox addition

When MSCs-*TetR*/*Amelx* were cultured in the presence of Dox, enhanced gene

expression of *Amelx* was confirmed after 24 hours (**Fig. 2A**). Western blotting analysis showed that Dox stimulated MSCs-*TetR*/*Amelx* to express amelogenin protein after 48 hours. In contrast, no background expression of amelogenin protein was observed when MSCs-*TetR*/*Amelx* were cultured in the absence of Dox (**Fig. 2B**).

3) Controllable expression of *Amelx* in MSCs-*TetR*/*Amelx* by addition and depletion of Dox

To confirm the controllable expression of *Amelx*, MSCs-*TetR*/*Amelx* were cultured in the osteogenic induction medium for 17 days in four different conditions. Condition 1: cells were cultured in the absence of Dox for 17 days, Condition II: cells were cultured in the absence of Dox until day 14, then cultured in the presence of Dox form day 15 to 17. Condition III: cells were cultured in the presence of Dox until day 14, then cultured in the absence of Dox form day 15 to 17. Condition IV : cells were cultured in the presence of Dox for 17 days.

A quantitative real-time RT-PCR analysis showed that expression of *Amelx* in cells from day 14 to 17 in the Condition IV was markedly higher than that in the Condition I (**Fig. 3**). In the Condition II, expression of *Amelx* significantly increased on day 15 (one day after Dox addition) (ANOVA; $P<0.01$). On the other hand, In the Condition III,

expression of *Amelx* markedly dropped after day 15 (one day after Dox depletion), and significantly decreased after day 16 (ANOVA; $P<0.01$). These results showed controllable expression of *Amelx* by addition or depletion of Dox.

In addition, a coincident expression pattern of *Osteocalcin* was observed in the four culture conditions. Expression of *Osteocalcin* was significantly increased by Dox addition within 72 hours (Condition II) (ANOVA; $P<0.01$); while sharply decreased by Dox depletion within 48 hours (Condition III) (**Fig. 4 A**). Expression of *Osterix* was also up-regulated after the Dox addition on day 14 (Condition II); while it significantly decreased by the Dox depletion within 72 hours (Condition III) (**Fig. 4 B**).

2. Effects of forced expression of *Amelx* on osteogenic differentiation of MSCs

1) Forced expression of *Amelx* enhanced expression of osteogenic marker genes

RT-PCR results showed that MSCs-*TetR*/*Amelx*, which were treated with Dox during osteogenic induction, increased gene expression of *Osterix*, *BSP* and *Osteocalcin* (**Fig. 5**). Quantitative real-time RT-PCR analysis demonstrated that the Dox addition significantly induced expression of *BSP* (**Fig. 6A**) and *Osterix* (**Fig. 6B**) genes after 14 days of osteogenic induction (ANOVA; $P<0.01$). Expression of *type I collagen* (**Fig. 6C**)

was also significantly enhanced by the Dox addition on day 14 (ANOVA; $P<0.01$). On the other hand, Dox did not stimulate expression of the osteogenic genes when MSCs-*TetR*/*Amelx* were cultured in the growth medium.

2) Forced expression of *Amelx* enhanced ALP activity and mineralized nodule formation of MSCs-*TetR*/*Amelx*

ALP staining results showed that the Dox addition clearly induced ALP activity of MSCs-*TetR*/*Amelx* after 7 days when the cells were cultured in the osteogenic induction medium (**Fig. 7A**). von Kossa staining results indicated that the Dox addition enhanced mineralized nodule formation of MSCs-*TetR*/*Amelx* after 21 days in the osteogenic induction medium (**Fig. 7B**).

Results of the Alizarin Red S staining also supported the enhanced matrix calcification of MSCs-*TetR*/*Amelx* by Dox addition (**Fig. 8A**). Quantitative analysis of the Alizarin Red S staining showed that the Dox addition significantly induced calcium deposition of MSCs-*TetR*/*Amelx* after 14 days in the osteogenic induction medium (ANOVA; $P<0.01$) (**Fig. 8B**).

3) Effects of forced expression of *Amelx* at different osteogenic differentiation stages on matrix calcification of MSCs

To examine effects of forced expression of *Amelx* on calcification of MSCs at different osteogenic differentiation stages, Dox was added to MSCs-*TetR*/*Amelx* at three different stages. When Dox was added on day 0-10 (early stage) of osteogenic differentiation, Alizarin Red S staining showed that calcium deposition significantly increased by the Dox addition (ANOVA; $P<0.01$) (**Fig. 9A**). In contrast, significant difference in calcium deposition by the Dox addition was not observed when Dox was added on day 10-20 (intermediate stage) or day 20-30 (late stage) of osteogenic differentiation (**Fig. 9B and 9C**). These results suggest that forced expression of *Amelx* at the early stage of osteogenic differentiation enhances matrix calcification of MSCs.

3. Establishment of a Tet-controlled *Amelx*/*Bmp4* expression system in iPSCs

1) Establishment of iPSCs-*TetR*

After the transduction of iPSCs with the pLenti3.3/*TetR* expression vector, drug selection with 500 μg/mL geneticin was performed. After the colony pick-up, eight clonal iPSC cultures were established (iPSC-clone 1-8). RT-PCR and Western blotting

results indicated that the iPSC-clone 6 (**Fig. 10A**) showed the highest expression of *TetR* (**Fig. 10B**); however, the expression level of *TetR* in the iPSC-clone 6 was significantly lower than that in the positive control of HEK293. Therefore, I attempted to increase the expression level of *TetR* by repeated transduction.

After additional 6-time transduction, the colony of iPSCs-*TetR* was selected with 10 mg/mL geneticin. After expansion of the colony, clonal iPSC culture, which sustained high ALP activity as pluripotent character, was established (**Fig. 10C**). RT-PCR and Western blotting analysis demonstrated that the iPSCs-*TetR* showed higher expression of *TetR* gene and protein compared to the iPSC-clone 6 that was subjected to the first transduction (**Fig. 10D**).

2) Establishment of iPSCs-*TetR*/*Amelx*

After the transduction of iPSCs-*TetR* with the pLenti6.3/TO/V5/*Amelx* expression vector, drug selection with 400 μg/mL blasticidin S was performed. A survived colony of iPSCs was picked up and expanded to establish iPSC-*TetR*/*Amelx* clonal culture (**Fig. 11A**). ALP staining showed that the iPSC-*TetR*/*Amelx* sustained robust ALP activity.

When iPSCs-*TetR*/*Amelx* were cultured in the presence of Dox in the growth medium, enhanced gene expression of *Amelx* was confirmed after 48 hours (**Fig. 11B**).

Western blotting analysis also detected induced expression of amelogenin protein by Dox addition after 48 hours; while little background expression of amelogenin was observed in the absence of Dox. Quantitative real-time RT-PCR analyses indicated that concentrations of Dox higher than 0.2 μg/mL significantly induced *Amelx* at 48 and 72 hours (ANOVA; $P<0.01$) (**Fig. 11C**). Therefore, Dox concentration of 0.2 μg/mL was used in the following experiments.

3) Effects of forced expression of *Amelx* on *in vivo* differentiation ability of iPSCs

When iPSCs-*TetR*/*Amelx* were cultured in the presence or absence of Dox for 21 days, both conditions of iPSCs-*TetR*/*Amelx* produced teratoma after 10 weeks of transplantation (**Fig. 12A**). Histological observation of the teratomas showed that both conditions of iPSCs-*TetR*/*Amelx* produced several types of tissues from all germ layers, such as cartilage (**Fig. 12B**), gut-like epithelium (**Fig. 12C**) and epidermis (**Fig. 12E and F**). However, a number of ectodermal epidermis-like tissues was significantly higher in the teratoma from the Dox treated iPSCs-*TetR*/*Amelx* compared to the teratoma from iPSCs-*TetR*/*Amelx* without Dox treatment (student-*t* test; $P<0.05$) (**Fig. 13A**). On the other hand, significantly lower number of mesodermal cartilage tissues and endodermal gut-like epithelia were observed in the teratoma from the Dox treated

iPSCs-*TetR*/*Amelx* compared to the teratoma from iPSCs-*TetR*/*Amelx* without Dox treatment (student-*t* test; $P<0.05$) (**Fig. 13B and C**).

4) Establishment of iPSCs-*TetR*/*Bmp4*

After the transduction of iPSCs-*TetR* with the pLenti6.3/TO/V5/*Bmp4* expression vector, drug selection with 400 μg/mL blasticidin S was performed. A survived colony of iPSCs was picked up and expanded to establish iPSC-*TetR*/*Bmp4* clonal culture (**Fig. 14A**). ALP staining showed that the iPSCs-*TetR*/*Bmp4* sustained robust ALP activity.

When iPSCs-*TetR*/*Bmp4* were cultured in the presence of Dox in the growth medium, enhanced gene expression of *Bmp4* was confirmed after 48 hours (**Fig. 14B**). Quantitative real-time RT-PCR analyses indicated that 0.2 μg/mL of Dox significantly induced *Bmp4* both at 48 and 72 hours (ANOVA; $P<0.01$) **(Fig. 14C)**.

5) Odontogenic differentiation of iPSCs by co-culture of *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs

When iPSCs-*TetR*/*Amelx* and iPSCs-*TetR*/*Bmp4* were co-cultured in the presence of Dox for 21 days, expression of *Amelx* significantly increased compared to those in the absence of Dox (ANOVA; $P<0.01$) (**Fig. 15A**). In addition, the co-cultured

iPSCs-*TetR*/*Amelx* and iPSCs-*TetR*/*Bmp4* in the presence of Dox showed significantly higher expression of *DMP1* than those in the absence of Dox on day 21 (ANOVA; $P<0.01$) (**Fig. 15B**).

Discussion

Amelogenin is comprised of three domains, N-terminal tyrosine-rich domain, central hydrophobic proline-rich domain, and a C-terminal hydrophilic telopeptide (61). Amelogenin gene sequence is present on both the human X and the human Y chromosomes, and exclusively on the mouse X chromosome (62). Therefore, production of the amelogenin protein in mouse is regulated by the *Amelx* (*amelogenin, X-linked*) gene. In this study, I successfully established a Tet-controlled *Amelx* gene regulation system for MSCs and iPSCs in which transcriptional activation of *Amelx* in MSCs was associated with enhanced osteogenic differentiation.

In this study, a Tet-dependent lentiviral transcriptional regulatory system [T-REx system (47)] was used to establish MSCs-*TetR*/*Amelx*. When the cells were cultured in the presence of Dox, production of amelogenin protein was induced within 48 hours. Although a little leaked gene expression of *Amelx* was detected by the RT-PCR assay, there was no background expression of amelogein protein when the cells were cultured in the absence of Dox. Indeed, many studies pointed that the leakiness in the Tet-dependent regulatory systems is quite unavoidable and acceptable in the most experimental cases (63-66).

Osteogenic differentiation of mineralizing cell types can be induced in the growth

medium supplement with dexamethasone, β-glycerophosphate and ascorbic acid (67). Many studies showed that enamel matrix derivative (EMD), the active component of Emdogain®, stimulates mineralizing cell types to increase ALP activity and production of *Osteocalcin*, *Osteopontin*, and *BSP* (68). In contrast, previous reports which showed the effects of recombinant amelogenin on osteogenic differentiation of mineralizing cell types are relatively contradictious. Matsuzawa *et al.* reported that mouse recombinant amelogenin up-regulated *Osteocalcin* and *type I collagen* mRNA levels in osteoblast cell line (ROS17/2.8 cells) (69). Zeichner-David *et al.* reported that mouse recombinant amelogenin induced *Osteocalcin* and *BSP* but down-regulated t*ype I collagen* in mouse periodontal ligament cells. In mouse cementoblasts, recombinant amelogenin or tyrosine-rich amelogenin peptide down-regulated *Osteocalcin* and *BSP* and inhibited mineral nodule formation (10, 70). Therefore, the effects of amelogenin on osteogenic differentiation may depend on the cell types and on the full-length or the domain-derived peptide.

Regarding the effects of amelogenin on MSCs, increased osteogenesis phenotype was reported after treatment with recombinant amelogenin (14, 71) or N-terminal amelogenin peptide (NTAP) (71). However, little is known about the effects of forced expression of *Amelx* gene on the osteogenesis, especially on the matrix mineralization.

In this study, I showed that forced expression of *Amelx* during osteogenic differentiation of MSCs clearly increased ALP activity and matrix calcification. In addition, the forced expression of *Amelx* leads to induction of mature osteogenesis of MSCs by up-regulating osteogenic genes, such as *Osterix*, *Osteocalcin*, *BSP*, and *type I collagen*. The observed up-regulation of these osteogenic genes should be due to the exogenous *Amelx* expression but not be due to the direct effect of Dox because Dox did not significantly alter the expression of the osteogenic genes in the original MSCs (data not shown).

In this study, I focused on the effects of forced expression of *Amelx* on MSCs which were undergoing osteogenic differentiation. In this sense, I first examined whether the expression of *Amelx* could be controlled by the Tet-dependent regulatory system even in the differentiation process of the MSCs. As a result, I found that the system could enhance/reduce the expression of *Amelx* by addition/depletion of Dox in the intermediate stage (day 14-17) of the osteogenic differentiation (**Fig. 3**).

Osteogenic differentiation of MSCs is a well-orchestrated process, beginning with activation of transcription factors including *Runx2* and *Osterix* (72). In the late stage of the osteoblast developmental sequence, *Osteocalcin* contributes as a regulator of the mineralization process (73). I next examined whether the controllable expression of

Amelx could concomitantly affect the expression of osteogenic genes, such as *Runx2*, *Osterix* and *Osteocalcin*. Interestingly, the expression of *Osterix* and *Osteocalcin* altered in parallel with the controlled expression of *Amelx* (**Fig. 4**). In contrast, expression of *Runx2* was not significantly affected by the controlled expression of *Amelx* (data not shown). It has been demonstrated that amelogenin also has cell signaling properties (71, 74-76). Amelogenin promotes osteogenic differentiation of MSCs through Wnt/beta-catenin signaling pathway by up-regulating *Wnt10b* expression (76). Olivares-Navarrete *et al.* recently reported that both amelogenin and NTAP induce osteogenic differentiation of human MSCs, and that the effects of the NTAP are mediated through PKC and ERK1/2 activation and β-catenin degradation (71). Shimizu *et al*. demonstrated that amelogenin stimulates *BSP* expression in osteoblasts through fibroblast growth factor 2 (FGF2) response element and transforming growth factor-β1 (TGF-β1) activation element in the promoter of the *BSP* gene (75). In this study, the concomitant expression of *Osterix* and *Osteocalcin* with the controlled expression of *Amelx* implies that the exogenous *Amelx* expression affects the transcriptional activation of *Osterix* and *Osteocalcin*. More work will be necessary to elucidate this possibility.

In this study, the *Amelx* transduction during the osteogenic differentiation enhanced osteogenesis of MSCs, which supports the positive effects of recombinant amelogenin

on osteogenic differentiation of MSCs (14, 71). The exogenous expression of *Amelx* significantly enhanced expression of *Osterix*, *type I collagen*, and *BSP* (**Fig. 5**). Tanimoto *et al.* reported that recombinant amelogenin enhanced osteogenic differentiation of human MSCs by up-regulating osteogenic genes including *ALP*, *BSP*, *type I collagen* and *Osteopontin* (14). Using human MSCs, Hu *et al.* performed genome-wide expression profiling to analyze the effects of lentiviral transduction of human Amelx on the gene regulation (15). They detected *Osteopontin*, *BMP-2*, *BMP-6*, and *VEGFC* as up-regulated genes by the forced expression of *Amelx*. In my study, forced expression of *Amelx* did not significantly alter the expression of *Osteopontin* (**Fig. 5**). This discrepancy likely results from differences in the species of MSCs and/or experimental conditions, that is, Hu *et al.* cultured the MSCs in the growth medium; whereas in this study the effects of *Amelx* transduction was performed during osteogenic induction in the osteogenic induction medium.

Under the osteogenic condition, approximately 2.5-fold expression of *Osterix* and *type I collagen* was stimulated by the exogenous *Amelx* expression at the intermediate stage (day 14) of the osteogenic differentiation (**Fig. 6B and 6C**). The exogenous *Amelx* expression also significantly induced the *BSP* expression after 14 days of osteogenic differentiation, and showed approximately 5.5-fold higher expression on day 21 (**Fig.**

6A). Osterix is a typical transcription factor required for osteoblast differentiation and bone formation (77). Type I collagen is a primary product of osteoblasts and its gene expression is observed at an early to intermediate stage during bone matrix formation (14, 78). Expression of *BSP* is detected in more differentiated osteoblasts at a relatively late stage (79). BSP is a non-collagenous protein component of mineralized tissues, such as cementum and bone, and is believed to be a critical molecule for promoting biomineralization (79, 80). From the standpoint of the roles of these molecules, the up-regulation of these genes by the *Amelx* transduction, at least in part, could contribute to the enhanced matrix calcification.

However, the 2.5- or 5.5- fold increased expression of these genes may not be sufficient to guide MSCs aggressively into more mature osteoblasts. Indeed, matrix calcification of MSCs already increased by the *Amelx* transduction on day 10 (**Fig. 9A**) although expression of osteogenic marker genes did not markedly increase until day 7 (**Fig. 5 and 6**). The full-length amelogenin has the capacity to stabilize the formation of amorphous calcium phosphate (ACP) (81). An abundant ACP phase is involved in a biomineralization in bone as a precursor phase that later transforms into the mature crystalline mineral (82, 83). In addition, Deshpande *et al.* recently demonstrated that amelogenin interacts with collagen fibrils and mineral particles to lead to mineralization

of collagen fibrils (84). Therefore, the increased ECM mineralization by the *Amelx* transduction would involve the direct effects of the resultant amelogenin protein on the ECM mineralization in addition to the effects of up-regulation of the osteogenic genes. The role of amelogenin in regulating the calcium phosphate mineral formation should mainly contribute in a precursor phase (the early stage) of biomineralization, so that explains the reason why the forced expression of *Amelx* did not significantly contribute to the calcification of MSCs when it started after the intermediate and late stages of the osteogenic differentiation (**Fig. 9B and 9C**).

Information from the present study, along with the Tet-dependent *Amelx* regulatory systems in MSCs, will help to clarify the mechanisms by which amelogenin regulates key molecules associated with mineralization. Defining the role for the amelogenin in controlling the activity of MSCs will provide the optimal application of amelogenin therapy for periodontal/bone regeneration.

iPSC is a well characterized stem cell that demonstrates pluripotency by contributing to lineages of cells and tissues from all three germ layers; therefore, the cell attracts attention in the field of regenerative medicine (85-88). In the field of dental research, iPSCs are expected to be used as a cell source for tooth regeneration (43). Because iPSCs have been shown to differentiate into ameloblasts (42, 89) and dental

mesenchymal cells (41, 89), the iPSC-derived dental cells may be useful to induce reciprocal epithelial-mesenchymal interactions to regulate the growth and morphogenesis of teeth. Recently, Cai *et al.* demonstrated that interaction of human iPSC-derived epithelial sheets and mouse embryo-derived dental mesenchymes led to generate tooth-like structures at the transplanted site of the mouse subrenal capsule (90). Liu *et al.* reported that mouse iPSCs could be induced to differentiate into ameloblast-like cells and odontoblast-like cells by ameloblasts serum-free conditioned medium (ASF-CM) and ASF-CM supplemented with BMP4, respectively (89). Based on these background, I attempted to develop an *in vitro* model of odontogenic guidance of iPSCs by the interaction of *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs.

Lentiviruses provide highly efficient gene delivery vehicles to constitutively express a regulatory protein for transgene expression in variety of cell types. Therefore, the lentiviral delivery systems have been utilized for the Tet-regulation system to achieve constitutive expression of *TetR* and target genes. However, inactivation of the virus promoter, such as cytomegalovirus (CMV), in ES cells after the gene delivery is well documented (91, 92). In addition, pluripotent stem cells (ES cells and iPSCs) permit lower levels of CMV genome entry into the nucleus compared to other differentiated cells (93, 94). Currently, most commercially available Tet-regulated

systems contain CMV as a promoter of the transgenes. The expression vectors used in this study also utilized the CMV promoter. Therefore, the most difficult hurdle I encountered to generate Tet-regulation system was how to avoid the naturally-occurring silencing of exogenous gene expression in iPSCs. That is, the transgene silencing after the transduction with the *TetR* expression vector into iPSCs is the first dilemma. To obtain stable expression of the transgene, iPSCs were subjected to the repeated transduction and the cell cloning by the drug selection for each transgene. Finally, Tet-controllable *Amelx* and *Bmp4* expression in iPSCs were available after 7-times, 3-times, and 2-times repeats of the process for *TetR*, *Amelx*, and *Bmp4*, respectively. The colonies of iPSCs-*TetR*/*Amelx* and iPSCs-*TetR*/*Bmp4* showed robust ALP activity, which is a pluripotent marker for ES cells and iPSCs. In addition, the teratoma formation assay demonstrated *in vivo* differentiation ability of iPSCs-*TetR*/*Amelx* into lineages of tissues from three germ layers. These results suggest that the iPSCs with the Tet-regulation system should, at least in part, possess pluripotent characteristics as pluripotent stem cells. As far as I know, this is the first report which achieved the Tet-controllable transgene expression in the undifferentiated state of iPSCs. However, it should be noted that the repeated transduction could result in multiple random genome insertions, which may lead to unexpected functional or structural alterations of the iPSCs. In order to

minimize the effects of silencing of the viral promoters, human elongation factor-1 α (EF1 α) promoters, which are recognized to show resistant ability to the silencing, should be used in the future studies.

Although amelogenin is originally identified as a protein of exclusively epithelial origin (95, 96), later studies demonstrated that amleogenins could be detected in odontoblasts (97, 98), indicating that amelogenin would be a biological molecule during odontogenic differentiation. In this study, teratoma assay showed that the *Amelx*-expressing iPSCs predominantly produced ectodermal epidermis-like tissues (**Fig. 12 and 13**). Expression of amelogenin is a characteristics of the epithelial odontogenic tumors (99). The results imply that constant *Amelx* expression during the EB culture of iPSCs stimulated cell signaling and guided their differentiation into epithelial cell lineages. In developing embryonic teeth, epithelial cells in the oral epithelium differentiate into ameloblasts. On the other hand, *Bmp4* is expressed in dental epithelium (100) and induces dental mesenchymal cell differentiation during early tooth development (33). I further examined whether the co-culture of the *Amelx*-expressing iPSCs and the *Bmp4*-expressing iPSCs stimulate the regulation of epithelial-mesenchymal interactions. The co-culture resulted in the increased expression of *Amelx* and *DMP1* (**Fig. 15**). Ameloblasts robustly express *Amelx* at the

secretory-stage of the differentiation process (101). Marked increase of *Amelx* expression on day 21 may be due to the effects of the forced expression of *Bmp4* because BMPs are known as factors that regulate mature differentiation of ameloblasts and induce secretion of amelogenin (102). DMP1 is considered to be an odontoblast marker, which is expressed in odontoblasts in the late stages of tooth development (103, 104). Therefore, these results implied that the experimental model using *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs stimulated odontogenic differentiation of iPSCs. Control of the regulation of epithelial-mesenchymal interactions by the Tet-regulated expression of odontogenic genes in iPSCs may prove to be a useful strategy for regulating odontogenesis and tooth regeneration.

Conclusions

1. Tet-controlled *Amelx* gene regulation systems for MSCs and iPSCs were successfully established.
2. Transcriptional activation of *Amelx* increases osteogenic differentiation of MSCs by up-regulating *Osterix*, *BSP*, *Osteocalcin* and *type I collagen* genes.
3. Interaction of *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs may lead odontogenic differentiation.

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Table 1: Primers used for RT-PCR analyses

Gene	**Primers** (Fw, forward; Rv, reverse)	**Ann Temp**	**Product size** (bp)	**Species**
TetR	Fw:5'- CGCCTTAGCCATTG GATGT-3' Rv:5'- TCTGCACCTTGGTGATCAAA-3'	54.5°C	390	mouse
Amelx	Fw:5'-CAGCAACCAATGATGCCAGTTCCT-3' Rv:5'- ACTTCTTCCCGCTTGGTCTTGTCT-3'	59.6°C	293	mouse
Bmp4	Fw: 5'-TCCACAGCACTGGTCTTGAG-3' Rv:5'- CGATCAACTAATCCTGACAT-3'	56.5°C	476	human
BSP	Fw:5'-AAAGTGAAGGAAAGCGACGA-3' Rv:5'-GTTCCTTCTGCACCTGCTTC-3'	58°C	214	mouse
Runx2	Fw: 5'-CGGGCTACCTGCCATCAC-3' Rv: 5'-GGCCAGAGGCAGAAGTCAGA-3'	65°C	289	mouse
Osterix	Fw: 5'-CTCGTCTGACTGCCTGCCTAG-3' Rv: 5'-GCGTGGATGCCTGCCTTGTA-3'	59°C	270	mouse
Oteocalcin	Fw: 5'-CCGGGAGCAGTGTGAGCTTA-3' Rv: 5'-AGGCGGTCTTCAAGCCATACT-3'	60°C	292	mouse
Osteopontin	Fw:5'-TCACCATTCGGATGAGTCTG-3' Rv:5'-ACTTGTGGCTCTGATGTTCC-3'	55°C	437	mouse
GAPDH	Fw: 5'-TGCACCACCAACTGCTTAG-3' Rv: 5'-GGATGCAGGGATGATGTTC-3'	67°C	418	mouse

Ann Temp: Annealing temperature

Table 2: Primers used for quantitative real-time RT-PCR analyses

Gene	**Primers** (Fw, forward; Rv, reverse) **Applied Biosystem Cat. #**	**Ann Temp**	**Product size** (bp)
Osterix	Fw: 5'-CTCGTCTGACTGCCTGCCTAG-3' Rv: 5'-GCGTGGATGCCTGCCTTGTA-3'	60°C	84
Col1agen 1a1	Fw: 5'-TGTCCCAACCCCCAAAGAC-3' Rv: 5'-CCCTCGACTCCTACATCTTCTGA-3'	60°C	92
Oteocalcin	Fw: 5'-CCGGGAGCAGTGTGAGCTTA-3' Rv: 5'-AGGCGGTCTTCAAGCCATACT-3'	60°C	68
GAPDH	Fw: 5'-TGCACCACCAACTGCTTAG-3' Rv: 5'-GGATGCAGGGATGATGTTC-3'	60°C	177
BSP	Mm00492555_m1		
Amelx	Mm01166221_m1		
Bmp4	Hs00370078_m1		
DMP1	Mm01208363_m1		
GAPDH	NM_008084/ Mm99999915_g1		

Ann Tmp: Annealing temperature

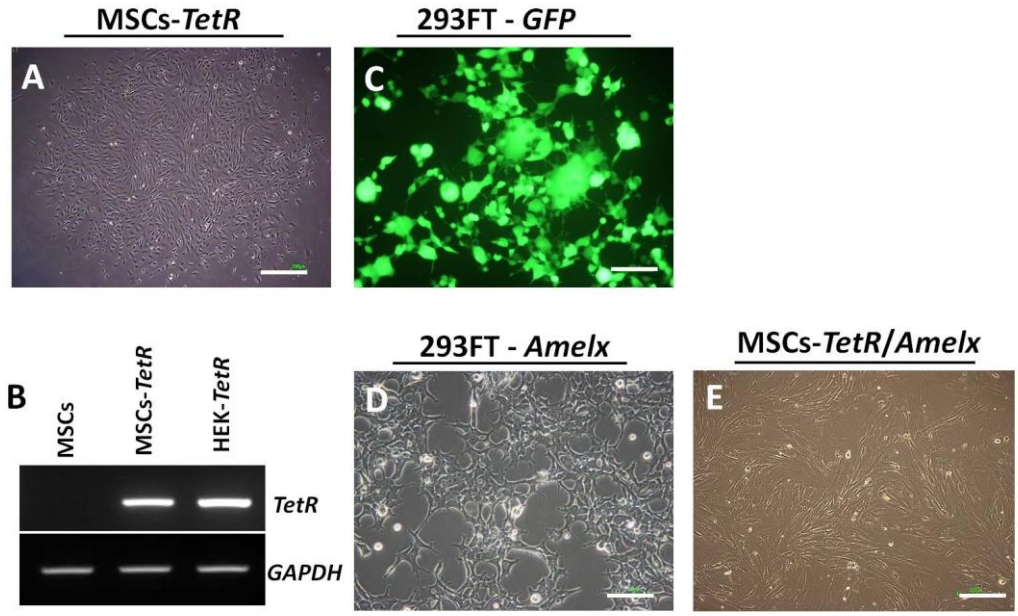


Fig. 1: Establishment of a Tet-controlled *Amelx* expression system in MSCs

A: A MSC colony (MSCs-*TetR*) in the culture medium containing 500 µg/mL geneticin 10 days after the transduction with the pLenti3.3/*TetR* expression vector. Bar, 60 µm.

B: Expression of TetR repressor gene in MSCs (without transduction) and MSCs-*TetR* was determined by RT-PCR. HEK293 cells with the same transduction procedure (HEK-*TetR*) were used as a positive control.

C: 293FT cells were introduced by the expression vector plenti6.3/V5-GW/*EmGFP* as control. After transfected 24 hours, GFP expressed strongly. Bar, 200 µm.

D: 293FT cells were introduced by the expression vector pLenti6.3/TO/V5/*Amelx* to produce *Amelx*-carring lentivirus. Bar, 200 µm.

E: MSCs-*TetR* were lentivirally transduced with the expression vector pLenti6.3/TO/V5/*Amelx*. MSCs-*TetR*/*Amelx* were selected by 10 µg/mL blastcidin S. Bar, 200 µm.

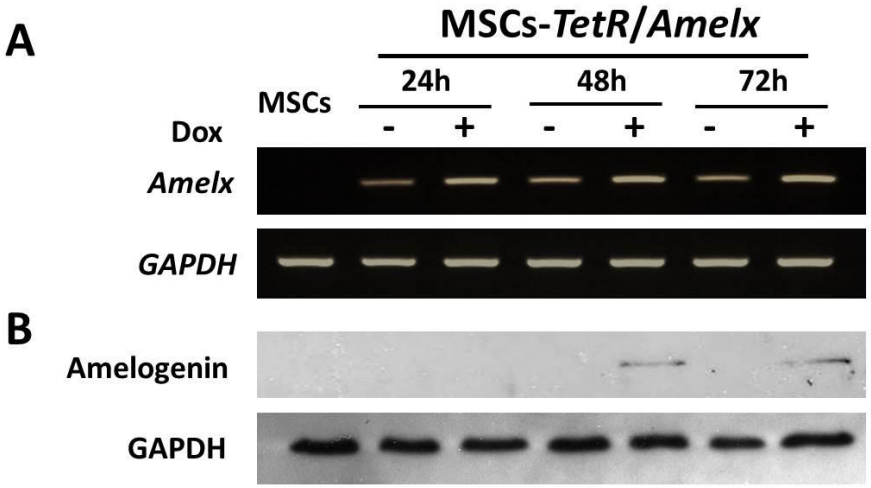


Fig. 2: Inducible expression of *Amelx* in MSCs-*TetR*/*Amelx* by Dox addition
MSCs-*TetR*/*Amelx* were cultured in the growth medium in the presence (+) or absence (-) of Dox for 24-72 hours. Inducible expression of *Amelx* gene was detected by RT-PCR (**A**) and Western blotting (**B**) analyses. GAPDH was used as a loading control.

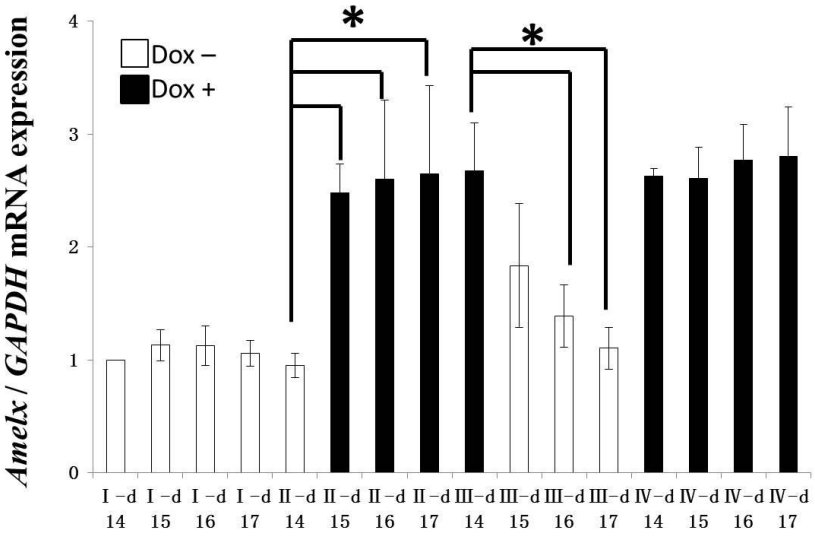


Fig. 3: Controllable expression of *Amelx* in MSCs-*TetR*/*Amelx* by Dox addition/depletion

MSCs-*TetR*/*Amelx* were cultured in the osteogenic induction medium in the presence (+) (black bars) or absence (-) (white bars) of Dox for 17 days in four different conditions. (Condition I: day 0-17 Dox-; Condition II: day 0-14 Dox-, day 15-17 Dox+; Condition III: day 0-14 Dox+, day 15-17 Dox-; Condition IV： day 0-17 Dox+). Expression of *Amelx* was determined by a quantitative real-time RT-PCR analysis. Significant differences (**P*<0.01, ANOVA with Tukey's multiple comparison test, n=4) were evaluated with respect to the values between the bars which were connected by a line.

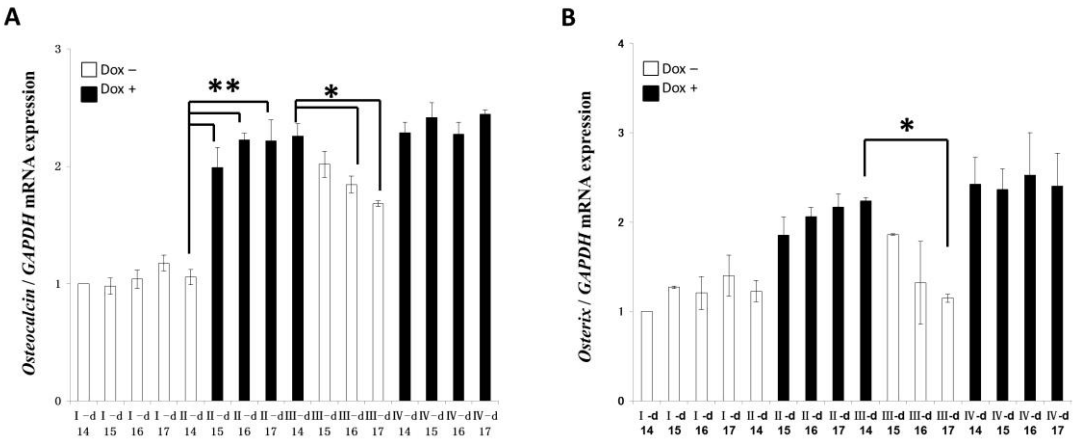


Fig. 4: Controllable expression of *Osterix* and *Osteocalcin* in MSCs-*TetR*/*Amelx* by Dox addition/depletion

MSCs-*TetR*/*Amelx* were cultured in the osteogenic induction medium in the presence (+) (black bars) or absence (-) (white bars) of Dox for 17 days in four different conditions. (Condition I: day 0-17 Dox-; Condition II: day 0-14 Dox-, day 15-17 Dox+; Condition III: day 0-14 Dox+, day 15-17 Dox-; Condition IV： day 0-17 Dox+). Expression of *Osteocalcin* (**A**) and *Osterix* (**B**) was determined by a quantitative real-time RT-PCR analysis. Significant differences (*P<0.01, ANOVA with Tukey's multiple comparison test, n=3) were evaluated with respect to the values between the bars which were connected by a line.

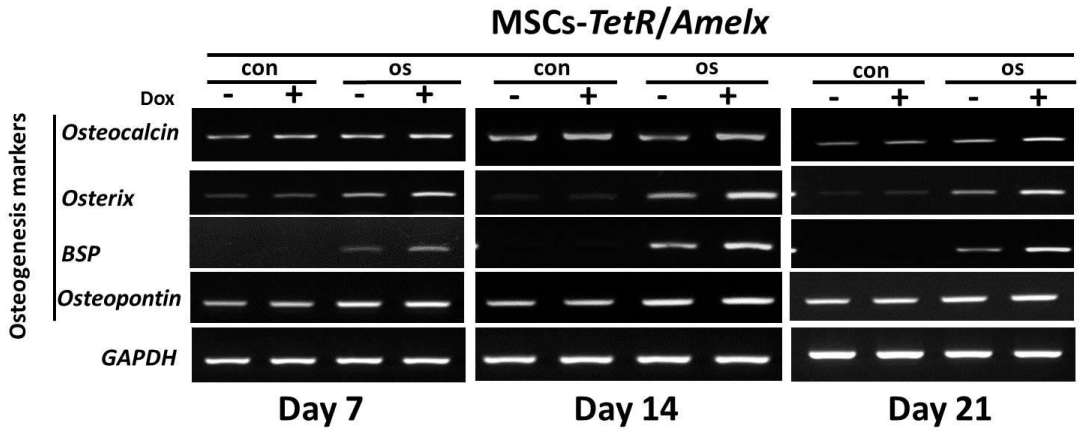


Fig. 5: Effects of forced expression of *Amelx* in MSCs-*tetR*/*Amelx* on expression of osteogenic marker genes

MSCs-*TetR*/*Amelx* were cultured in growth medium (con) or osteogenic induction medium (os) in the presence (+) or absence (-) of Dox for 21 days. The expression of osteogenesis marker genes (*Osteocalcin*, *Osterix*, *BSP* and *Osteopontin*) was examined by RT-PCR analysis. *GAPDH* was used as a loading control.

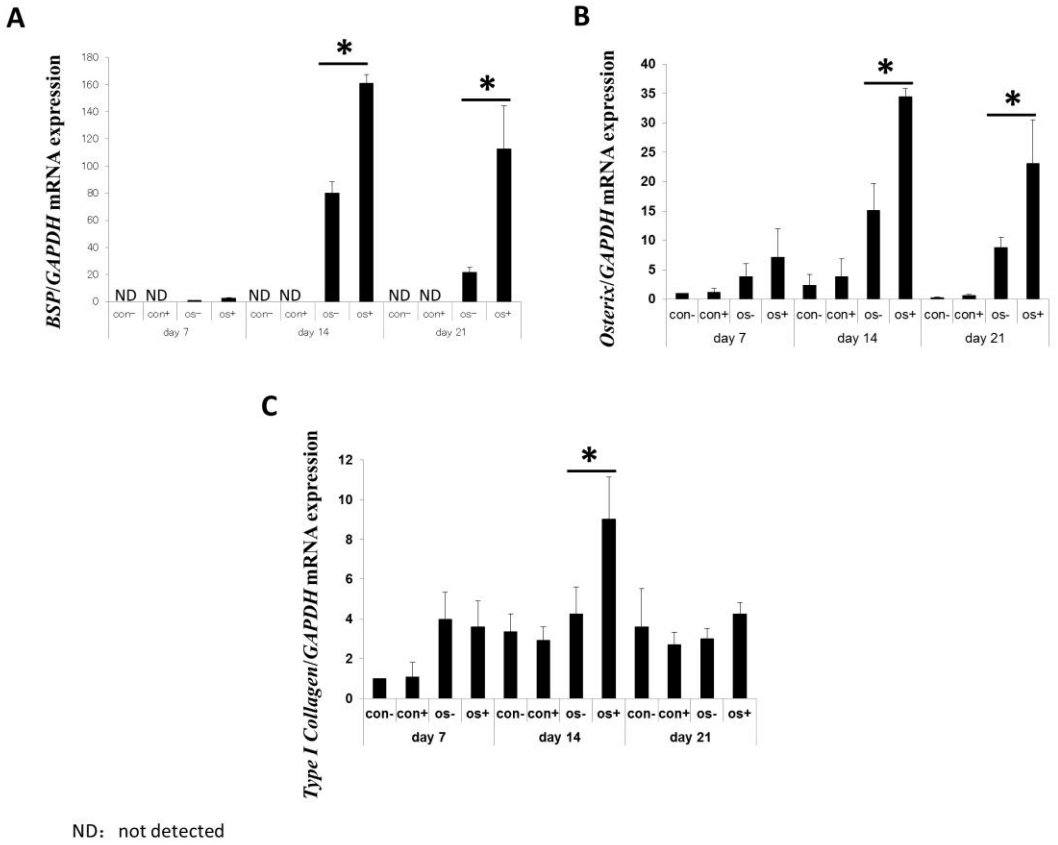


Fig.6: Effects of forced expression of *Amelx* in MSCs-*tetR*/*Amelx* on expression of osteogenic marker genes

MSCs-*TetR*/*Amelx* were cultured in growth medium (con) or osteogenic induction medium (os) in the presence (+) or absence (-) of Dox for 21 days. The expression of *BSP* (**A**), *Osterix* (**B**) and *type I collagen* (**C**) genes were examined by quantitative real-time RT-PCR analysis. *GAPDH* was used as an internal control. Significant differences (**P*<0.01, ANOVA with Tukey's multiple comparison test, n=4 for *type I collagen*, n=3 for *BSP* and *Osterix*) were evaluated with respect to the values between Dox (+) and (-), which were connected by a line.

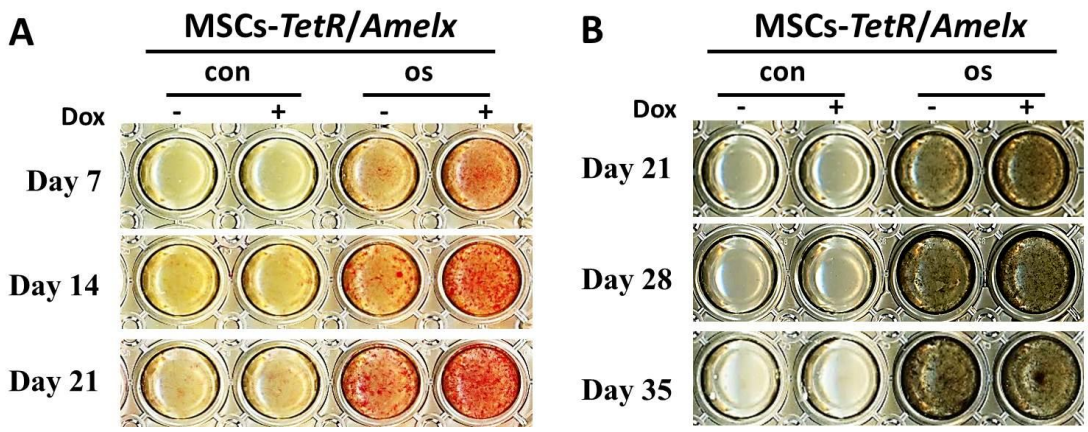


Fig.7: Effects of forced expression of *Amelx* on the ALP activity and mineralized nodule formation

MSCs-*TetR*/*Amelx* were cultured in the growth medium (con) or the osteogenic induction medium (os) in the presence (+) or absence (-) of Dox.

A: ALP activity on day 7, 14 and 21 was examined by ALP staining.

B: Mineralized nodule formation on day 21, 28 and 35 was detected by von Kossa staining.

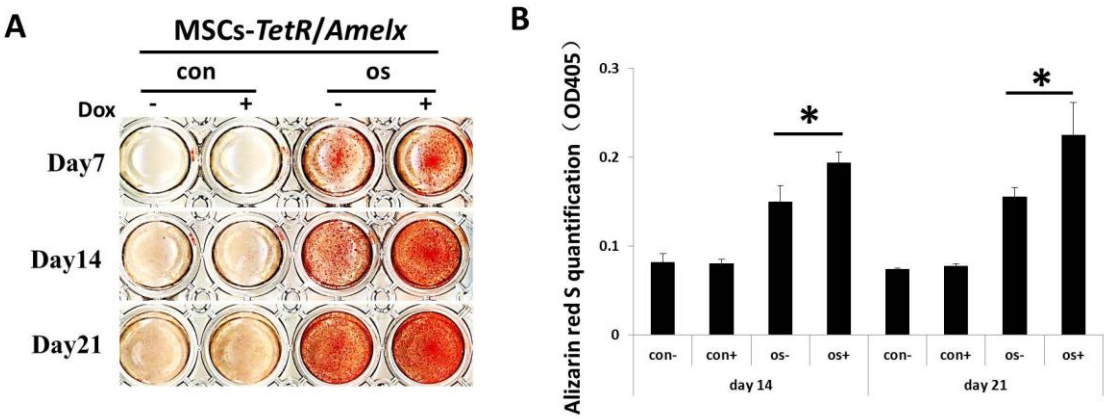


Fig.8: Effects of forced expression of *Amelx* on the calcium deposition

MSCs-*TetR*/*Amelx* were cultured in the growth medium (con) or the osteogenic induction medium (os) in the presence (+) or absence (-) of Dox for 21 days. Calcium deposition was determined by Alizarin Red S staining (**A**) and the quantitative analysis of the staining intensity (**B**). Significant differences (**P*<0.01, ANOVA with Tukey's multiple comparison test, n=9) were evaluated with respect to the values between Dox (+) and (-) on day 14 and 21.

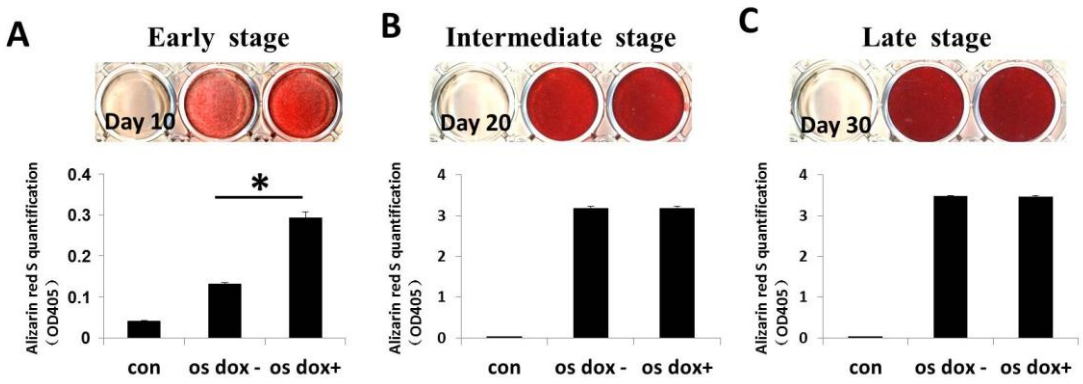


Fig.9: Effects of forced expression of *Amelx* at different osteogenic differentiation stages on matrix calcification of MSCs

MSCs-*TetR*/*Amelx* were cultured in the growth medium (con) or the osteogenic induction medium (os). Dox was added to MSCs-*TetR*/*Amelx* on day 0-10 (**A**: early stage), day 10-20 (**B**: intermediate stage), or day 20-30 (**C**: late stage) of osteogenic differentiation, and Alizarin Red S staining was performed at day 10, day 20, or day 30, respectively. Quantitative analysis of the staining intensity was also performed. Significant differences (*P<0.01, ANOVA with Tukey's multiple comparison test, n=9) were evaluated with respect to the values between Dox (+) and (-).

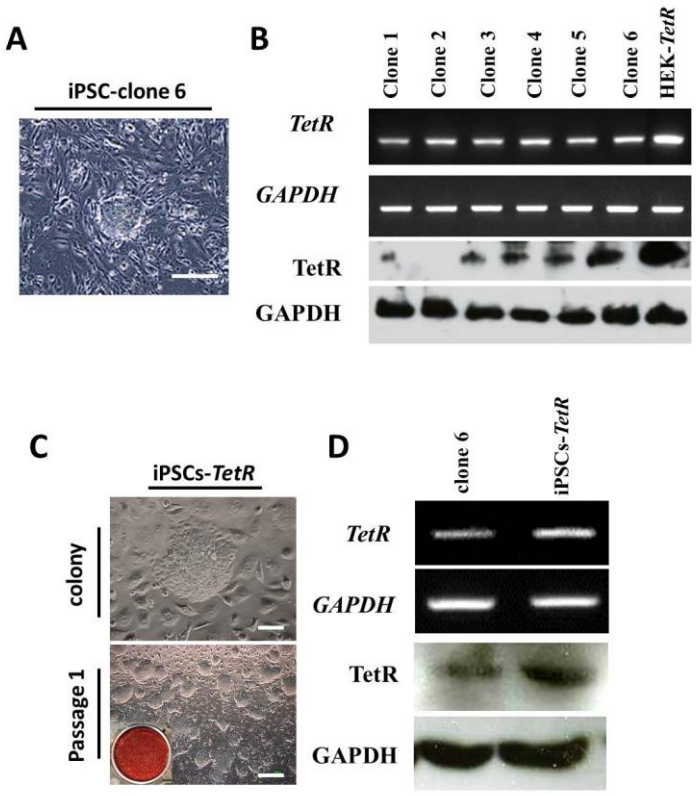


Fig. 10: Establishment of iPSCs-*TetR*

A: Mouse gingiva-derived iPSCs were lentivirally infected with *TetR* expression vector. Eight clonal cultures were established after the drug selection with 500 μg/mL geneticin. The picture shows an iPSC colony of one of the clonal cultures (iPSC-clone 6). Bar, 40 μm.

B: Expression of *TetR* gene and protein expression in the eight clonal iPSC cultures (clone 1-8) were examined by RT-PCR and Western blotting analyses. HEK293 cells with the same transduction procedure (HEK-*TetR*) were used as a positive control.

C: After additional 6-time transduction, a survived iPSC colony (upper panel) with 10 mg/ml geneticin was picked up. Clonal culture (iPSCs-*TetR*: lower panel) was established from the colony. Inset: ALP staining image of iPSC-*TetR*. Bar, 40 μm.

D: Expression of *TetR* gene and protein expression in the iPSCs after one-time transduction (clone 6) and seven-time transduction (iPSCs-*TetR*) were examined by RT-PCR and Western blotting analysis.

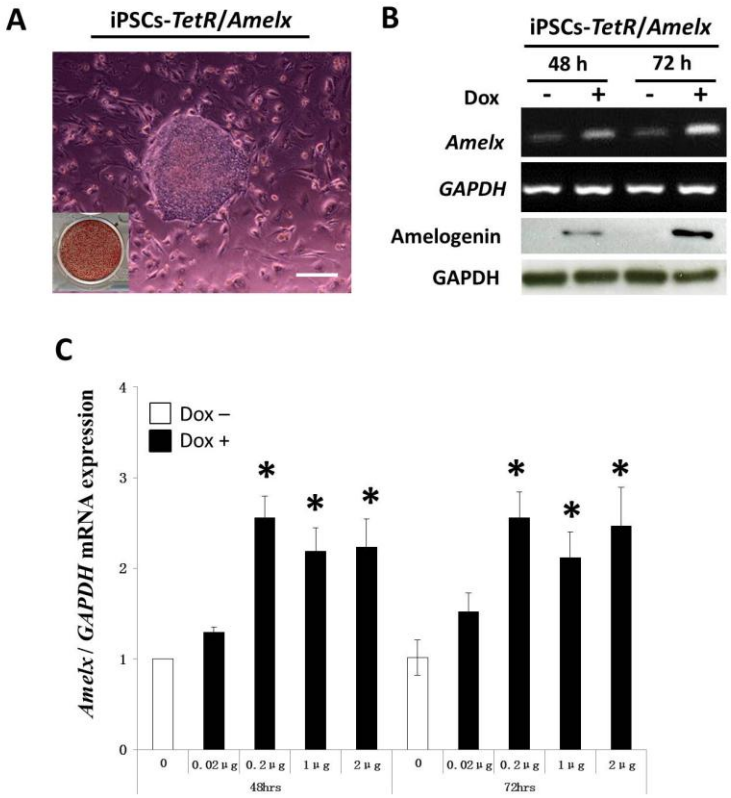


Fig. 11: Establishment of iPSCs-*TetR*/*Amelx*

A: iPSCs-*TetR* were transduced with pLenti6.3/TO/V5/*Amelx* expression vector. After a drug selection with 400 μg/mL blastcidin S, clonal iPSC culture was established (iPSCs-*TetR*/*Amelx*). Inset: ALP staining image of the iPSCs-*TetR*/*Amelx*. Bar, 40 μm.

B: iPSCs-*TetR*/*Amelx* were cultured in the ES medium in the presence (+) or absence (-) of Dox for 72 hours. *Amelx* gene and protein expression were observed by RT-PCR and Western blotting analyses. GAPDH was used as a loading control.

C: iPSCs-*TetR*/*Amelx* were cultured in the ES medium in the presence (+) (black bars) or absence (-) (white bars) of Dox (0.02μg/mL, 0.2μg/mL, 1μg/mL, 2μg/mL) for 72 hours. Expression of *Amelx* gene was examined by quantitative real-time RT-PCR analysis. *GAPDH* was used as an internal control. Significant differences (**P*<0.01, ANOVA with Tukey's multiple comparison test, n=3) were evaluated with respect to the values between Dox (+) and (-) at the same time point.

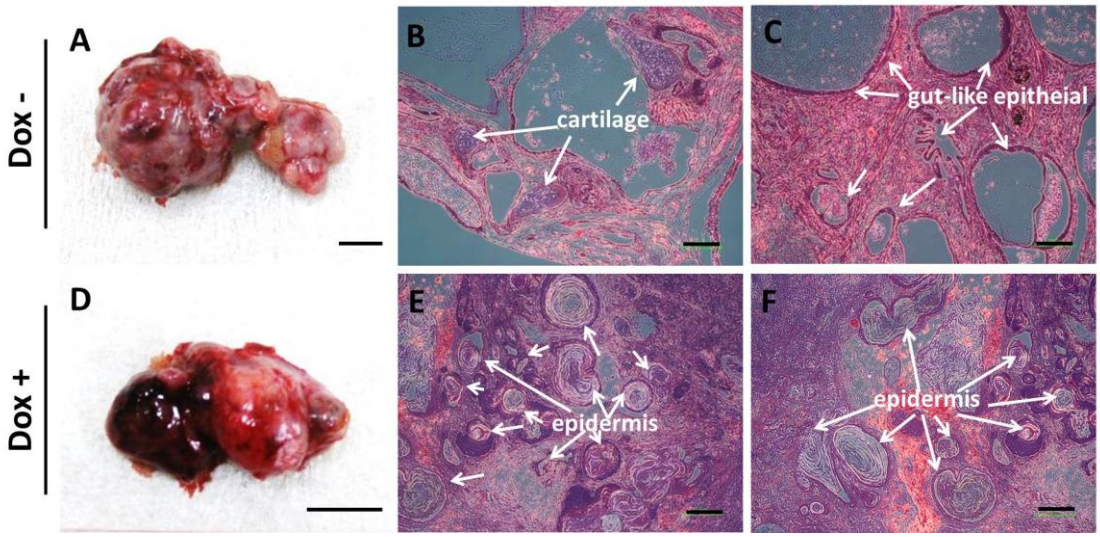


Fig. 12: Effects of forced expression of *Amelx* on *in vivo* differentiation ability of iPSCs

iPSCs-*TetR*/*Amelx*, which were cultured in the presence (Dox+) (**D-F**) or absence (Dox-) (**A-C**) of Dox, were transplanted under the testis of a mouse. After 10 weeks of transplantation, both conditions of iPSCs-*TetR*/*Amelx* produced teratoma (**A, B**: bars, 1 cm). Histological observation (H&E staining) of the teratoma showed that both conditions of iPSCs-*TetR*/*Amelx* produced several types of tissues from all germ layers, such as cartilage (**B**: arrows), gut-like epithelium (**C**: arrows) and epidermis (**E, F**: arrows). Bars, 200 μm.

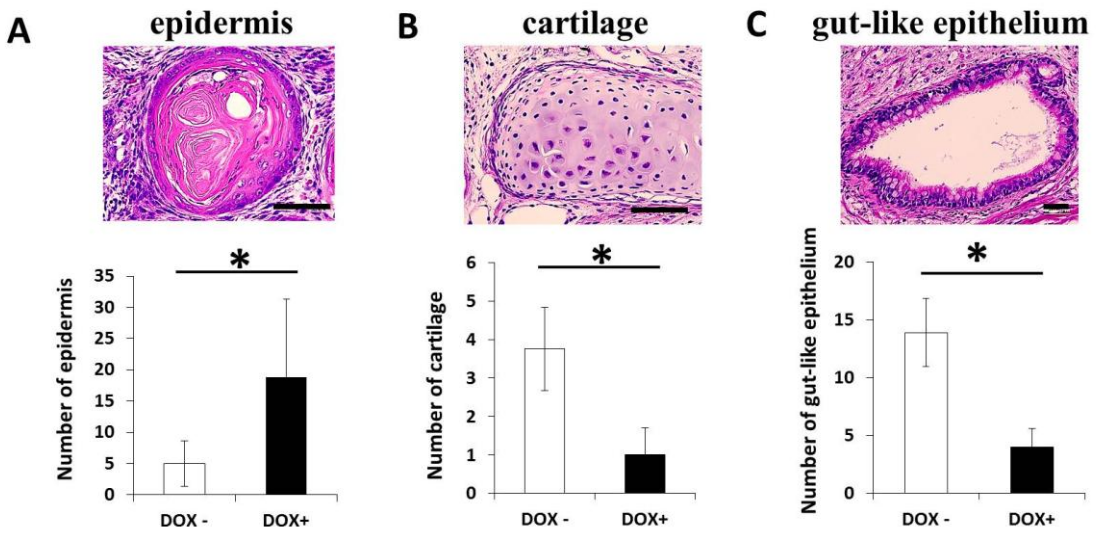


Fig. 13: Forced expression of *Amelx* guided differentiation of iPSCs into epidermis *in vivo*

iPSCs-*TetR*/*Amelx*, which were cultured in the presence (Dox+) or absence (Dox-) of Dox, were transplanted under the testis of a mouse. After 10 weeks of transplantation, histological observation (H&E staining) of the teratoma was performed, and a number of epidermis (**A**), cartilage (**B**), or gut-like epithelium (**C**) in the histological section was counted. Significant differences (**P*<0.05, student-T test: n=8) were evaluated with respect to the values between Dox (+) and (-). Bars, 100μm.

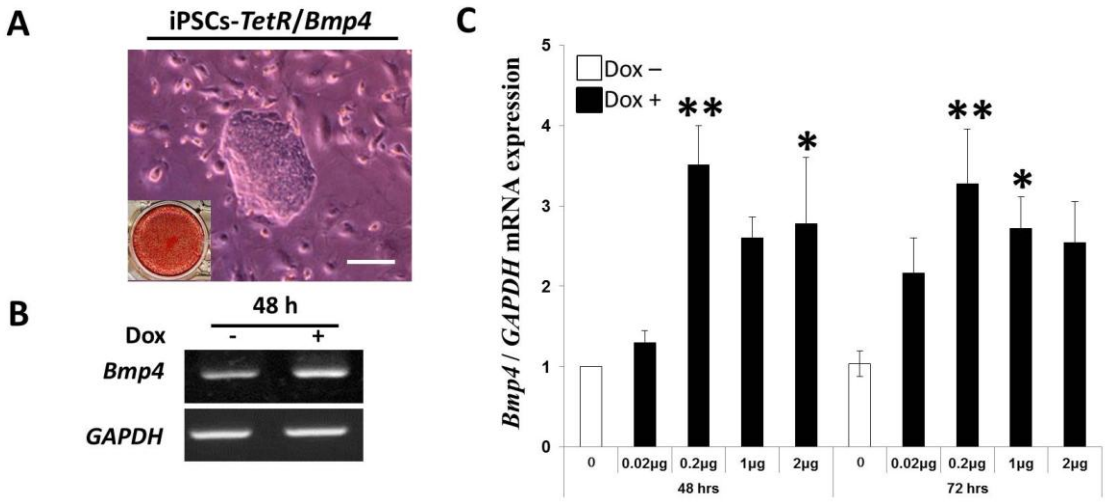
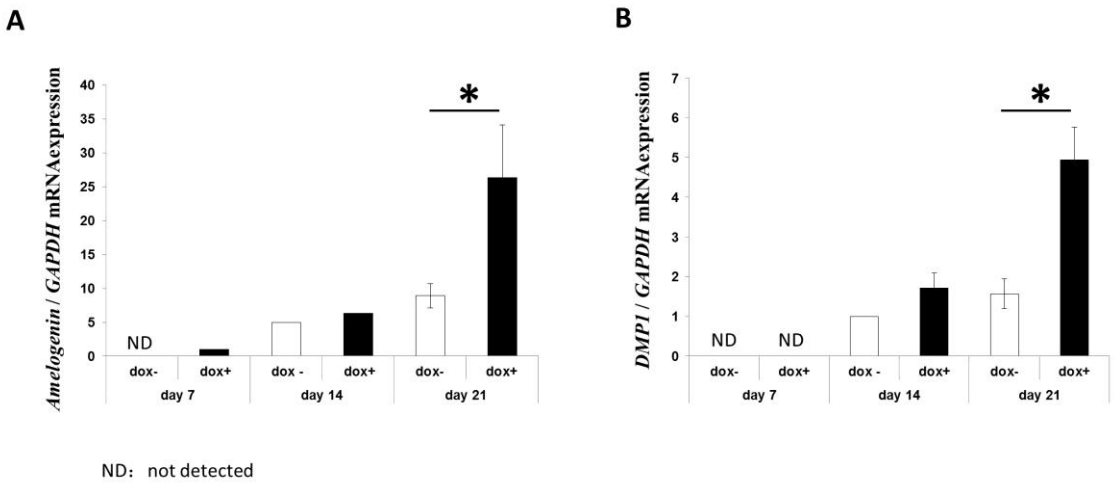


Fig. 14: Establishment of iPSCs-*TetR*/*Bmp4*

A: iPSCs-*TetR* were transduced with pLenti6.3/TO/V5/*Bmp4* expression vector. After a drug selection with 400 μg/mL blastcidin S, clonal iPSC culture was established (iPSCs-*TetR*/*Bmp4*). Inset: ALP staining image of the iPSCs-*TetR*/*Bmp4*. Bars, 40 μm.

B: iPSCs-*TetR*/*Bmp4* were cultured in the ES medium in the presence (+) or absence (-) of Dox for 48 hours. Expression of *Bmp4* gene was determined by RT-PCR analysis. *GAPDH* was used as a loading control.

C: iPSCs-*TetR*/*Bmp4* were cultured in the ES medium in the presence (+) (black bars) or absence (-) (white bars) of Dox (0.02 μg/mL, 0.2 μg/mL, 1 μg/mL, 2 μg/mL) for 72 hours. Expression of *Bmp4* gene was examined by quantitative real-time RT-PCR analysis. *GAPDH* was used as an internal control. Significant differences (**P<0.01，*P<0.05, ANOVA with Tukey's multiple comparison test, n=3) were evaluated with respect to the values between Dox (+) and (-) at the same time point.



ND：not detected

Fig. 15: Odontogenic differentiation of iPSCs by co-culture of *Amelx*-expressing iPSCs and *Bmp4*-expressing iPSCs

EBs of iPSCs-*tetR*/*Amelx* and iPSCs-*tetR*/*Bmp4* were co-cultured in the presence (+) (black bars) or absence (-) (white bars) of Dox for 21 days. Expression levels of (**A**) *Amelx* (ameloblast-lineage) and (**B**) *DMP1* (odontoblast-lineage) were evaluated by quantitative real-time RT-PCR analyses. Significant differences (**P*<0.01, ANOVA with Tukey's multiple comparison test, n=3) were evaluated with respect to the values between Dox (+) and (-).