



Title	Controlled Differentiation of Stem Cells by Tetracycline-Controlled Transcriptional Activation of Amelogenin
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## 論文内容の要旨

氏名(王放放)	
論文題名	Controlled Differentiation of Stem Cells by Tetracycline-Controlled Transcriptional Activation of Amelogenin
論文内容の要旨	
<p><b>Introduction</b></p> <p>Amelogenin is the primary component of Emdogain®, which is used to regenerate periodontal defects prior to prosthodontic treatments. Amelogenin is well known for its effect on nucleation/growth of hydroxyapatite crystals during enamel bio-mineralization. In addition, recent studies suggest that amelogenin has a unique function to modulate osteogenic differentiation of mesenchymal stem cells (MSCs); however, molecular mechanisms by which amelogenin contributes to stem cells remain unknown.</p> <p>Amelogenin is also known to be involved in the organizing of enamel rods during tooth development. Tooth development is largely dependent on sequential and reciprocal epithelial-mesenchymal interactions. Bone morphogenetic protein-4 (Bmp4) is one of the key mesenchymal odontogenic signals for driving tooth morphogenesis. Induced pluripotent stem cells (iPSCs) have potential to differentiate into dental epithelial and mesenchymal stem cells; therefore, controlled expression of amelogenin and Bmp4 in iPSCs may provide a new strategy to generate a bioengineered tooth.</p> <p>To investigate direct effects of amelogenin on stem cells, forced expression of amelogenin by the viral transduction is one of the most powerful and cost-effective methods. Tet-controlled transcriptional activation is a method of inducible gene expression where transcription is reversibly turned on or off in the presence of a tetracycline (Tet) derivative (doxycycline: Dox). In this study, I focused on the Tet-dependent lentiviral transcriptional regulatory system to control forced expression of exogenous amelogenin and Bmp4 genes in stem cells.</p> <p>The objectives of this study were first to establish a Tet-regulated system for amelogenin expression in MSCs and iPSCs, and second to investigate effects of exogenous expression of amelogenin on osteogenesis of MSCs, and third to explore a possibility to induce a bioengineered tooth by interaction of amelogenin expressing iPSCs and Bmp4 expressing iPSCs.</p>	
<p><b>Material and method</b></p> <p><b>1. Tet-controlled transcriptional activation of amelogenin during MSC osteogenesis</b></p> <p>I first investigated whether Tet-controlled transcriptional activation of amelogenin enhanced osteogenesis of MSCs. Immortalized clonal mouse bone marrow-derived MSCs were lentivirally infected with the pLenti3.3/TetR expression vector, and transduced cells (MSCs-TetR) were selected by geneticin. A full-length mouse amelogenin-coding (Amelx) cDNA fragment was PCR-amplified and cloned into pENTR™/D-TOPO vector using pENTR Directional TOPO cloning kit (Invitrogen). Based on this entry vector, pLenti6.3/Tet operator (TO)/V5/-Amelx expression vector that contained the Amelx cDNA fragment was constructed using the Gateway® system and lentivirally introduced into MSCs-TetR to generate MSCs-TetR/Amelx.</p> <p>Tet-dependent expression of Amelx gene and protein in MSCs-TetR/Amelx was evaluated by RT-PCR and Western blotting in the presence or absence of Dox for 7-35 days. Tet-dependent osteogenic marker gene [Osterix, Osteocalcin, Osteopontin, Bone sialoprotein (BSP), Type I collagen] expression, alkaline phosphatase (ALP) activity, mineralized nodule formation and calcium deposits were evaluated by RT-PCR (semi-quantitative RT-PCR and quantitative real-time RT-PCR analyses), ALP staining, von Kossa (VK) staining and quantitative alizarin red measurement assay, respectively.</p>	

## 2. Tet-controlled transcriptional activation of amelogenin and Bmp4 in iPSCs

I next investigated whether Tet-controlled transcriptional activation of amelogenin and Bmp4 induced odontogenesis of iPSCs. Mouse gingiva-derived iPSCs were lentivirally infected with the pLenti3.3/TetR expression vector, and transduced cells (iPSCs-TetR) were selected by geneticin. Expression of TetR gene in iPSCs-tetR was evaluated by RT-PCR and western blotting. A full-length human Bmp4 cDNA was cloned into pLenti6.3/TO/V5 expression vector (pLenti6.3/TO/V5-Bmp4) using the Gateway® system. After that, iPSCs-tetR were transduced by pLenti6.3/TO/V5-Amelx and pLenti6.3/TO/V5-Bmp4 to generate iPSCs-tetR/Amelx and iPSCs-tetR/Bmp4, respectively. Tet-dependent expression of Amelx and Bmp4 gene and protein in iPSCs-tetR/Amelx and iPSCs-tetR/Bmp4 were evaluated by RT-PCR and Western blotting in the presence or absence of Dox.

To investigate whether forced expression of amelogenin in iPSCs showed lineage-specific differentiation, embryonic bodies (EBs) of iPSCs-tetR/Amelx were cultured in the presence or absence of Dox, and were implanted under the skin of the mouse. After 10 weeks, implanted tissues were extracted and histologically examined by hematoxylin and eosin (HE) staining. EBs of iPSCs-tetR/Amelx and iPSCs-tetR/Bmp4 were co-cultured in the presence or absence of Dox for 21 days, and expression of ameloblast-lineage marker gene (amelogenin) and odontoblast-lineage marker gene (dentin matrix acidic phosphoprotein 1: DMP1) was examined by RT-PCR.

## Results

### 1. Tet-controlled transcriptional activation of amelogenin enhanced MSC osteogenesis

When MSCs-TetR/Amelx were cultured in the presence of Dox, enhanced gene expression of Amelx was confirmed after 24 hours. Western blotting analysis showed that Dox stimulated MSCs-TetR/Amelx to express Amelx protein after 48 hours. In contrast, no background expression of Amelx protein was observed when MSCs-TetR/Amelx were cultured in the absence of Dox. Corresponding to the increased expression of Amelx with the addition of Dox, osteogenesis marker genes (osterix, BSP, osteocalcin and type I collagen) expressed significantly higher than the cells cultured without Dox (ANOVA;  $P<0.01$ ). Forced expression of Amelx increased ALP activity after 14 days and the mineralized nodule formation after 21 days during osteogenic differentiation. Quantitative analysis of calcification demonstrated that forced expression of Amelx significantly induced calcium deposition of MSCs-TetR/Amelx after 14 days until 21 days in osteogenic induction medium (ANOVA;  $P<0.01$ ).

### 2. Tet-controlled transcriptional activation of amelogenin and Bmp4 induced odontogenic differentiation of iPSCs

Enhanced gene and protein expression of Amelx and Bmp4 was detected after 48 hours in the presence of Dox in iPSCs-tetR/Amelx and iPSCs-tetR/Bmp4, respectively. Histological observation of the transplanted iPSCs-tetR/Amelx indicated that the cells predominantly formed keratinocyte-like cells and epidermis-like tissues in the presence of Dox, while the cells cultured in the absence of Dox formed teratoma that characteristically had cells and tissues from all three germ layers. Co-culture of EBs of iPSCs-tetR/Amelx and iPSCs-tetR/Bmp4 in the presence of Dox showed significantly higher expression of amelogenin and DMP1 compared to those in the absence of Dox (ANOVA;  $P<0.01$ ).

## Conclusions

These results suggest that Tet-controlled Amelx gene regulation systems for MSCs and iPSCs were successfully established. Transcriptional activation of Amelx enhances osteogenic differentiation of MSCs by up-regulating osterix, BSP, osteocalcin and type I collagen genes. Interaction of amelogenin expressing iPSCs and Bmp4 expressing iPSCs may lead odontogenic differentiation. These findings represent important steps toward the optimal application of amelogenin therapy for periodontal/bone regeneration and the new strategy for a bioengineered tooth.

## 論文審査の結果の要旨及び担当者

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## 論文審査の結果の要旨

本研究は、テトラサイクリン依存性の *Anelogenin* 転写制御が可能なマウス間葉系幹細胞および iPS 細胞を樹立し、これらを用いて幹細胞の分化制御について検討したものである。

その結果、マウス間葉系幹細胞における *Anelogenin* 遺伝子の強制発現は骨芽細胞分化を促進することが示された。また、マウス iPS 細胞に *Anelogenin* あるいは *BMP-4* 遺伝子を強制発現させて相互作用させることで、iPS 細胞を歯原性細胞に誘導できる可能性が示唆された。

本研究は、再生歯学における骨の再生および歯の再生の研究に貴重なデータを提供するものであり、博士（歯学）の学位授与に値するものと認める。