J Mol Hist (2016) 47:375–387
DOI 10.1007/s10735-016-9680-5

ORIGINAL PAPER

Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) expression and possible function in mouse tooth germ development

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Received: 18 March 2016 / Accepted: 18 May 2016 / Published online: 27 May 2016

Abstract Abnormal expression of Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) is involved in the pathogenesis of FSHD. FRG1 is also important for the normal muscular and vascular development. Our previous study showed that FRG1 is one of the highly expressed genes in the mandible on embryonic day 10.5 (E10.5) than on E12.0. In this study, we investigated the temporospatial expression pattern of FRG1 mRNA and protein during the development of the mouse lower first molar, and also evaluated the subcellular localization of the FRG1 protein in mouse dental epithelial (mDE6) cells. The FRG1 expression was identified in the dental epithelial and mesenchymal cells at the initiation and bud stages. It was detected in the inner enamel epithelium at the cap and early bell stages. At the late bell and root formation stages, these signals were detected in ameloblasts and odontoblasts during the formation of enamel and dentin matrices, respectively. The FRG1 protein was localized in the cytoplasm in the mouse tooth germ in vivo, while FRG1 was detected predominantly in the nucleus and faintly in the cytoplasm in mDE6 cells in vitro. In mDE6 cells treated with bone morphogenetic protein 4 (BMP4), the protein expression of FRG1 increased in cytoplasm, suggesting that FRG1 may translocate to the cytoplasm. These findings suggest that FRG1 is involved in the morphogenesis of the tooth germ, as well as in the formation of enamel and dentin matrices and that FRG1 may play a role in the odontogenesis in the mouse following BMP4 stimulation.

Keywords FRG1 · Tooth germ · Tooth development · Expression pattern · Intracellular localization

Electronic supplementary material The online version of this article (doi:10.1007/s10735-016-9680-5) contains supplementary material, which is available to authorized users.

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Introduction

Odontogenesis is regulated by the sequential and reciprocal epithelial–mesenchymal interactions, similar to processes in other organs, e.g., hair follicles, salivary glands, lungs, kidneys (Thesleff 1995). Many reports regarding the complex multi-step process of gene expression during odontogenesis have been accumulating. These studies have demonstrated that various genes encoding signaling molecules, such as bone morphogenetic proteins (BMPs), fibroblast growth factor, Sonic hedgehog, Wnt families, Nfic, Pax6, extracellular matrix metalloproteinase inducer, four and a half LIM domains 2, and Jun activation domain-binding protein 1 are related to tooth morphogenesis (Thesleff and Åberg 1999; Cobourne and Sharpe 2003; Pispa

and Thesleff 2003; Thesleff 2003; Chen et al. 2009; Li et al. 2011; Chen et al. 2014; Lei et al. 2014; Xie et al. 2015; Du et al. 2016; Lian et al. 2016). However, the precise molecular signaling pathways related to the initiation, morphogenesis, differentiation and mineralization of the tooth germ have not yet been fully elucidated. Therefore, it is important to identify the regulatory genes that are involved in the signal networks activated during the tooth development.

We previously performed cDNA subtraction between mouse mandibles collected on embryonic day 10.5 (E10.5) and E12.0 to identify genes that might be involved in the development of the tooth germ (Yamaza et al. 2001a). Several dozen genes were found to be differentially expressed on E10.5 or E12.0, some of which were confirmed to be related to tooth development (Yamaza et al. 2001b; Wada et al. 2002; Akhter et al. 2005; Takahashi et al. 2010; Honda et al. 2011; Ookuma et al. 2013; Shiotsuka et al. 2013; Kihara et al. 2014). Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) is one of the genes highly expressed on E10.5, and is related to actin binding and angiogenesis (Wuebbles et al. 2009; Liu et al. 2010; Sun et al. 2011).

FRG1 is a candidate causative gene for FSHD (Grewal et al. 1998), which is the third most common muscular dystrophy. This disease leads to progressive atrophy of the muscles of the face, shoulders and upper arms. While FSHD is primarily a disease of the skeletal muscle, up to 75 % of FSHD patients also present with vascular defects (Fitzsimons et al. 1987; Padberg et al. 1995; Osborne et al. 2007). FRG1 is required for the normal development of the vertebrate musculature and vasculature (Hanel et al. 2009; Wuebbles et al. 2009), and is an actin-bundling protein associated with muscle attachment sites (Liu et al. 2010). Overexpressed FRG1 was shown to be localized in nucleoli, Cajal bodies and actively transcribed chromatin (van Koningsbruggen et al. 2007; Sun et al. 2011), thus indicating a role for FRG1 in RNA processing (Hanel et al. 2011). FRG1 is a dynamic nuclear and cytoplasmic shuttling protein (Hanel et al. 2011). Although FRG1 has been suggested to function in the development of the musculature and vasculature, little is known about the function of FRG1 during the initiation, morphogenesis, differentiation and mineralization of the tooth germ.

In this study, we demonstrate the FRG1 mRNA and protein expression during the development of the tooth germ of the mouse lower first molar from initiation to root formation and eruption. Furthermore, the subcellular localization of the FRG1 protein was investigated in mouse dental epithelial cells stimulated with BMP4, which has been reported to be a critical factor in odontogenesis (Åberg et al. 1997). Our present findings indicate that FRG1 may play a potential role in tooth development.

Materials and methods

Animals

BALB/c embryos and postnatal mice were used in this study. We used samples obtained on E10.5, 12, 14, 15, 16 and 18 after gestation, and on postnatal day 0 (P0), P1, P3, P10 and P20 to investigate the FRG1 expression pattern during the different developmental stages of the tooth. Adult mice were mated overnight. The embryonic day was defined as E0.5 based on the presence of a vaginal plug after allowing 12 h for the mating (Ookuma et al. 2013; Shiotsuka et al. 2013). Adult BALB/c mice were obtained from Charles River Japan Inc. (Yokohama, Japan). All manipulations of mice were approved by the Animal Care and Use Review Committee, Kyushu University (Fukuoka, Japan), and were performed in accordance with the guidelines of the committee.

In situ hybridization

Specific antisense cRNA probes for FRG1 mRNA were designed according to the NCBI Reference Sequences (accession number FRG1: NM_013522.3). A template cRNA probe for FRG1 mRNA was prepared using PCR amplification with the primer pairs described below, and was subcloned into the pGEM3Z vector (Promega, Madison, WI). These probes were labeled with digoxigenin (DIG)-UTP using the DIG RNA Labeling Kit (Roche, Mannheim, Germany). The gene-specific primers for FRG1 were as follows: 5′-CCC AAG CTT ATG GCC GAA TAT TCC TAT GTA AAG TCC-3′ and 5′-CCG GAA TTC TCA CTT GCA GTA TCG GTC AGC-3′.

A membrane hybridization was performed to confirm the specificity and sensitivity of the cRNA probes (Shiotsuka et al. 2013; Kihara et al. 2014). In situ hybridization was carried out according to our previous studies (Yamaza et al. 2001b; Wada et al. 2002; Akhter et al. 2005; Kobayashi et al. 2006; Xie et al. 2007; Honda et al. 2008; Xie et al. 2009; Akhter et al. 2010; Takahashi et al. 2010; Honda et al. 2011; Ookuma et al. 2013; Shiotsuka et al. 2013; Kihara et al. 2014). The embryos and mandibles of the postnatal mice were fixed in 4 % paraformaldehyde (PFA) in diethylpyrocarbonate-treated phosphate buffered saline (PBS, pH 7.4) overnight at 4 °C. After fixation, P3–P20 mandibles were additionally demineralized with 10 % ethylenediamine tetra-acetic acid (pH 7.4) at 4 °C for 3 days to 2 weeks depending on the tooth developmental stage. The samples were then embedded in OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan) and cut into serial frontal cryosections at 8-μm thickness. The cryosections were mounted on silane-coated glass slides

(Muto pure chemicals, Tokyo, Japan). After hybridization, the DIG-labelled probes were visualized by an alkaline phosphatase-conjugated anti-DIG antibody using BM Purple (Roche Molecular Biochemicals).

FRG1 sense probes were used as a negative control. "Strong" and "weak" signals were used to assess the relative signal intensity in the same tissue section.

Immunohistochemistry

For the immunohistochemical analysis, the serial cryosections were prepared in the same way as for in situ hybridization. To block nonspecific immunoreactions, the cryosections were incubated with 10 % normal goat serum (Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature (RT). The slides were incubated with primary antibodies, a rabbit polyclonal FRG1 IgG (diluted 1:800, FL-258; Santa Cruz Biotechnology, Dallas, TX), a rabbit monoclonal vascular endothelial growth factor receptor 2 (VEGFR2) IgG (diluted 1:800, 55B11; Cell Signaling Technology, Denver, MA) and a rabbit polyclonal BMP4 IgG (diluted 1:300, Abcam, Cambridge, UK), at 4 °C overnight. The sections were then reacted with Alexa Fluor 568 goat anti-rabbit IgG (diluted 1:2000, A11011; Invitrogen, Carlsbad, CA) or Alexa Fluor 488 goat anti-rabbit IgG (diluted 1:2000, A11034; Invitrogen) as a secondary antibody for 30 min at RT. Finally, the immunostained sections were counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Dojindo, Kumamoto, Japan). The sections were rinsed in PBS three times between the different steps.

In the in situ hybridization and immunohistochemical examinations, the tooth developmental stages were defined according to previous studies (Akhter et al. 2005; Shiotsuka et al. 2013; Kihara et al. 2014).

Cell line and cell culture

Mouse dental epithelial (mDE6) cells (Ookuma et al. 2013; Shiotsuka et al. 2013; Kihara et al. 2014) were maintained in DMEM/F-12 (Invitrogen) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5 % CO_2 at 37 °C.

Immunocytochemistry

To study the subcellular localization of the FRG1 protein, mDE6 cells treated with or without BMP4 (Wako, Osaka, Japan) were immunostained. The mDE6 cells were seeded onto an 8-well Permanox plastic chamber slides (Thermo, Yokohama, Japan; 1×10^4 cells/well) and were cultured in DMEM/F-12 supplemented with 10 % FBS. At 24 h after seeding, the cells were exposed to 5 ng/ml BMP4 for 24 h. After this BMP4 treatment, the cells were fixed in 4 % PFA in PBS for 10 min on ice. After fixation, cells were permeabilized with PBS containing 0.1 % Triton-X-100 (Wako) for 5 min on ice, and were blocked with 10 % normal goat serum (Nichirei) to prevent non-specific binding for 30 min at RT. The cells were incubated with primary antibodies against FRG1 (rabbit monoclonal anti-FRG1, 1:800, sc-135066; Santa Cruz Biotechnology) and β-actin (mouse monoclonal anti-β-actin, 1:500, A5441, Sigma-Aldrich, St. Louis, MO) at 4 °C overnight. These antibodies were visualized using an Alexa Fluor 488 goat anti-rabbit antibody (1:2000, A11034; Invitrogen) and Alexa Fluor 594 goat anti-mouse antibody (1:2000, A11005; Invitrogen) for 30 min at RT, respectively. Cell nuclei were counterstained with DAPI for 15 min at RT. Images were obtained with a Microscope Digital Camera System, DP71 (Olympus, Inc., Tokyo, Japan).

Western blot analysis

The proteins were extracted from mDE6 cell. Whole cell protein was generated according to our previous study (Ookuma et al. 2013). Nuclear and cytoplasmic fractions were isolated using the Readypre™ Protein Extraction Kit (Cytoplasmic/nuclear) (Bio-Rad, Hercules, CA). Western blot analysis was carried out according to the protocol described previously (Kiyoshima et al. 2014). Protein samples (20 μg) were separated by 12.5 % SDS-polyacrylamide gel (SDS-PAGE) and then transferred to Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad). After blocking, the membrane was incubated with rabbit monoclonal anti-FRG1 (sc-135066; Santa Cruz Biotechnology) and goat polyclonal anti-GAPDH (sc-20357; Santa Cruz Biotechnology) at 4 °C overnight. Bound antibodies were reacted with a 1:2000 dilution of HRP-conjugated secondary antibodies and visualized using the ECL Prime Western Blotting Detection system (GE Healthcare, Little Chalfont, UK). Emitted light was detected using the ImageQuant LAS 4000 (GE Healthcare), a cooled CCD-camera. In the semi-quantitative analyses of the levels of protein expression, the intensity of the bands was measured using the ImageQuant TL software (GE Healthcare). GAPDH was used an internal control protein. The target protein/GAPDH ratio based on the intensity of the bands was estimated, as previously described (Ookuma et al. 2013).

Statistical analysis

All experiments were independently performed at least three times. The one-way ANOVA with the Tukey–Kramer comparison test was used to analyze the data of western

blot analysis. Differences resulting in a P value of <0.01 were considered to be statistically significant.

Results

Expression pattern of FRG1 mRNA and protein in the developing mouse tooth germ

The in situ hybridization and the immunohistochemical analyses were performed to characterize the temporal and spatial expression of FRG1 mRNA and protein in the developing mouse lower first molar from E10.5 to P20.

Initiation (E10.5)

On this embryonic day, thickening of the oral mucosal epithelium to form the tooth bud was not yet observed. The signal of FRG1 mRNA and protein was diffusely detected in the epithelial layer and mesenchymal cells (Fig. 1a, b, d). However, some strong signals for the FRG1 protein in the mesenchyme appeared to be located in the vascular endothelial cells (Fig. 1b, d, yellow arrowhead), because the strongly FRG1-expressing cells were also positive for vascular endothelial growth factor receptor 2 (VEGFR2) (Fig. S1).

Thickening of the dental epithelium (E12)

On E12, the thickening of the oral mucosal epithelium was seen in a limited area, indicating tooth bud formation. The expression of FRG1 mRNA and protein was diffusely detected in the oral mucosal epithelium and mesenchymal cells (Fig. 1e, f, h). Some strong signals were found in the vascular endothelial cells (Fig. 1f, h, yellow arrowhead).

Bud stage (E14)

The tooth bud was formed when the thickened dental epithelium invaginated into the mandibular ectomesenchyme. The expression of FRG1 mRNA was observed in the epithelial cells of the tooth germ and in the mesenchymal cells surrounding the tooth bud on E14 (Fig. 1i).

On E14, the expression pattern of the FRG1 protein was similar to that of FRG1 mRNA at the bud stage (Fig. 1j, l). The intensity of the FRG1 protein expression in these cells was weaker than that in the vascular endothelial cells that were positive for VEGFR2 (Fig. S1, yellow arrowhead).

Cap stage (E15–16)

The tooth bud developed to become a cap-shaped structure with the primary enamel knot (PEK) in the center of the enamel organ on E15.

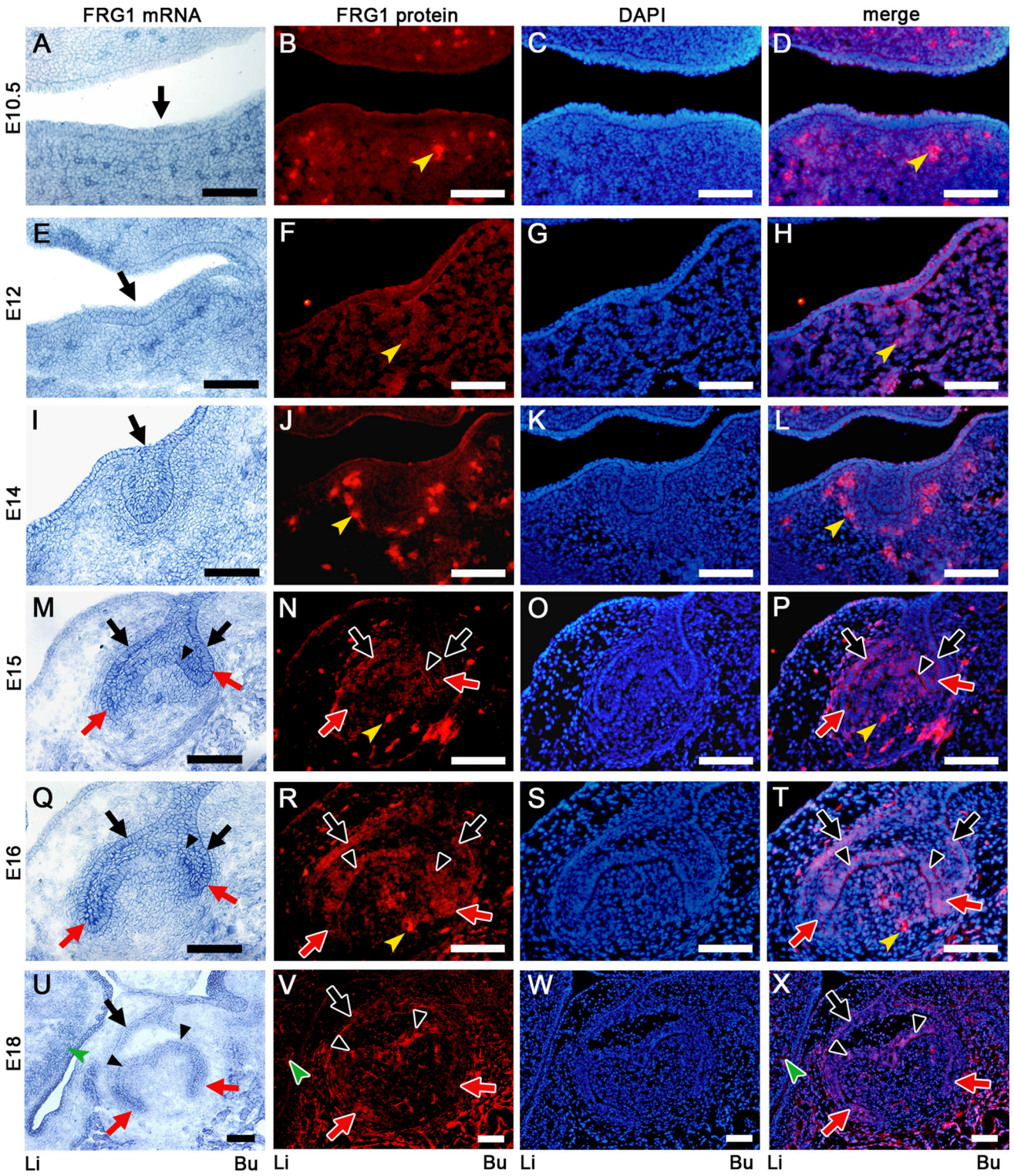
Fig. 1 The expression of FRG1 mRNA and protein in the tooth germ on E10.5–18. **a**, **e** On E10.5 and E12, the in situ FRG1 signals were diffusely detected in the epithelial layer (*black arrow*) and mesenchymal cells at the predicted lower first molar region. **b**, **d**, **f**, **h** The FRG1 protein was observed in the epithelial layer and mesenchyme. **i**, **j**, **l** At the bud stage (E14), the expression of FRG1 mRNA and protein was observed in the epithelial tooth bud (*black arrow*) and the surrounding mesenchymal cells. **m**, **q** At the cap stage (E15 and E16), a strong in situ FRG1 signal was detected in the outer enamel epithelium (*black arrows*), inner enamel epithelium (*black arrowhead*), PEK and in the cervical loop (*red arrows*). **n**, **p**, **r**, **t** Strong expression of the FRG1 protein was detected in the outer enamel epithelium (*black arrows*), inner enamel epithelium (*black arrowheads*), PEK and the cervical loop (*red arrows*). **u**, **v**, **x** At the early bell stage (E18), the expression of FRG1 mRNA and protein was found in the outer enamel epithelium (*black arrow*), inner enamel epithelium (*black arrowheads*), the cervical loop (*red arrows*), the stratum intermedium and in the muscular tissue of the tongue (*green arrowhead*). **c**, **g**, **k**, **o**, **s**, **w** The nuclei were counterstained with DAPI. *Yellow arrowheads* indicate some strong signals for the FRG1 protein in the vascular endothelial cells located in the mesenchyme. *Li*; lingual side, *Bu*; buccal side. *Scale bars*; 50 μm (**a–l**), 100 μm (**m–x**)

On E15, strong FRG1 mRNA expression was observed in the whole enamel organ including PEK. The dental papilla and dental sac also showed weak signals for FRG1 mRNA (Fig. 1m). On E16, extension of the cervical loop of the enamel organ appeared to begin. Although the expression pattern of FRG1 mRNA on E16 was similar to that on E15, strong FRG1 mRNA expression was noted in the cervical loop on E16 (Fig. 1q, red arrows).

At the cap stage, the expression pattern of FRG1 protein was almost the same as that of FRG1 mRNA. On E15, FRG1 protein expression was observed in the whole enamel organ including PEK. The intensity became similar to that in the vascular endothelial cells. The signal was also found in the dental papilla cells and dental sac (Fig. 1n, p). On E16, strong FRG1 protein expression was still detected in the outer enamel epithelium, inner enamel epithelium and cervical loop in the enamel organ, while the central cells of the enamel organ showed a decrease in the FRG1 protein expression (Fig. 1r, t). The intensity of the FRG1 protein signal in the dental papilla cells and dental sac appeared to be reduced compared with that on E15 based on the intensity of the FRG1 protein signal in the vascular endothelial cells, which was used as an internal control (Fig. 1n, p, r, t). Some signals were also found in the vascular endothelial cells around the enamel organ on E15 and E16 (Fig. 1n, p, r, t, yellow arrowhead).

Early bell stage (E18)

The cervical loop of the enamel organ extended to the mesenchymal tissue and enclosed the dental papilla. The tooth germ exhibited a bell-shaped structure at this stage.


FRG1 mRNA
FRG1 protein
DAPI
merge
E10.5
E12
E14
E15
E16
E18
A
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C
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The in situ signal of FRG1 was strongly expressed in the inner enamel epithelium, stratum intermedium and cervical loop (Fig. 1u, red arrows). The FRG1 mRNA signal in the inner enamel epithelium (black arrowheads) was stronger than that observed in the outer enamel epithelium (black arrow) (Fig. 1u). Weak in situ expression of FRG1 was observed in the dental papilla on E18 (Fig. 1u). The signal intensity was stronger in the cells facing the inner enamel epithelium than in the other cells in the dental papilla.

Immunofluorescent signals for FRG1 were observed particularly in the inner enamel epithelium, stratum intermedium and cervical loop on E18 (Fig. 1v, x). A weak signal was expressed in the outer enamel epithelium, dental sac and cells of the dental papilla facing the inner enamel epithelium on E18 (Fig. 1v, x).

There were no apparent differences between the protein signal and the in situ signal for FRG1 on E18. The expression of FRG1 mRNA and protein were also detected in the muscular tissue of the tongue, which was used as an internal control, on E18 (Fig. 1u, v, x, green arrowhead).

Late bell stage (P0–P3)

The inner enamel epithelium differentiated into ameloblasts and the dental pulp cells facing the inner enamel epithelium differentiated into odontoblasts at this stage. The cells in the stage just before matrix formation are termed preameloblasts and preodontoblasts, respectively (Byers et al. 1990; Shiotsuka et al. 2013). Neither enamel nor dentin matrices had formed on P0, while the formation of both enamel and dentin matrices between ameloblasts and odontoblasts was observed in the P1 and P3 tooth germs.

On P0, strong expression of FRG1 mRNA was detected in the inner enamel epithelium at the central groove region and in the preameloblasts and preodontoblasts at the lingual cusp site (Fig. 2a, e, g), while this was not observed in the cells of the dental papilla facing the inner enamel epithelium at the central groove region where matrix formation was not observed (Fig. 2a, k, m). The strong signal for the FRG1 protein was also localized in the inner enamel epithelium at the central groove region, and in the preameloblasts and preodontoblasts at the lingual cusp site where it almost started forming matrices (Fig. 2b, f, g, l, m).

On P1, strong FRG1 mRNA expression was detected in the ameloblasts and odontoblasts at the lingual cusp site (Fig. 2c, h, j) and in the preameloblasts and preodontoblasts at the central groove region (Fig. 2c, n, p). The in situ signal for FRG1 mRNA was also found in the stratum intermedium. Strong FRG1 protein expression was observed in the preameloblasts and preodontoblasts in the central groove region (Fig. 2d, o, p), although FRG1 protein expression was not noted in the cells of the dental papilla facing the inner enamel epithelium on P0 (Fig. 2b, l, m). Strong FRG1 protein expression was also observed in the ameloblasts and odontoblasts at the cusp site (Fig. 2d, i, j).

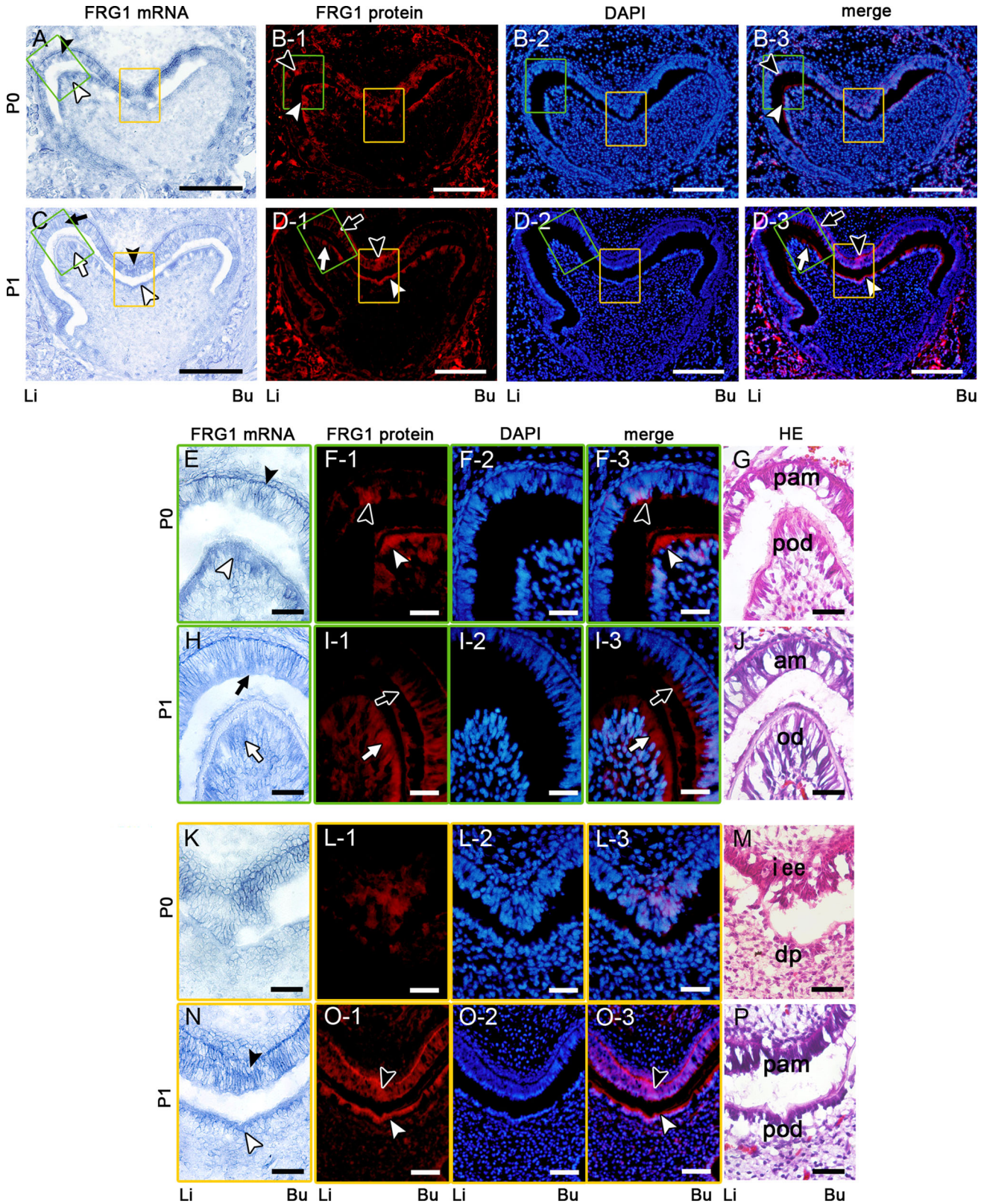
Fig. 2 The expression of FRG1 mRNA and protein in the tooth germ on P0 and P1. **a**, **b** On P0, strong expression of FRG1 mRNA and protein was detected in the inner enamel epithelium, and in the preameloblasts (*black arrowhead*) and preodontoblasts (*white arrowhead*) at the lingual cusp site. **c, d** On P1, strong expression of FRG1 mRNA and protein was observed in the ameloblasts (*black arrow*) and odontoblasts (*white arrow*) at the lingual cusp site, and in the preameloblasts (*black arrowhead*) and preodontoblasts (*white arrowhead*) at the central groove region. **e**, **k** The *green-* and *yellow-boxed areas* in (**a**) are respectively shown at a higher magnification. **f**, **l** The *green-* and *yellow-boxed areas* in (**b**) are respectively shown at a higher magnification. **h**, **n** The *green-* and *yellow-boxed areas* in (**c**) are respectively shown at a higher magnification. **i**, **o** The *green-* and *yellow-boxed areas* in (**d**) are respectively shown at a higher magnification. **g**, **j**, **m**, **p** The HE-stained images correspond to (**e**) & (**f**), (**h**) & (**i**), (**k**) & (**l**) and (**n**) & (**o**), respectively. *pam*; preameloblasts, *pod*; preodontoblasts, *am*; ameloblasts, *od*; odontoblasts, *iee*; inner enamel epithelium, *dp*; dental papilla, *Li*; lingual side, *Bu*; buccal side. *Scale bars*; 20 μm (**e–p**), 100 μm (**a–d**)

On P3, the in situ FRG1 signal was strongly expressed in preodontoblasts on the lateral sides of the tooth germ (white arrowhead) and odontoblasts (white arrow) (Fig. 3a, b). The in situ FRG1 signal was also detected in the preameloblasts (black arrowhead) and ameloblasts (black arrow) (Fig. 3a, b). A strong FRG1 protein signal was detected in the ameloblasts (black arrow) and odontoblasts (white arrow) (Fig. 3d, f), similar to that on P1. The FRG1 protein was strongly expressed in the preameloblasts (black arrowhead) and preodontoblasts (white arrowhead), especially on the lateral sides of tooth crown (Fig. 3g). Immunofluorescent and in situ signals were also found in the stratum intermedium (Fig. 3b, g, blue arrowhead).

Tooth root formation stage (P10)

The enamel matrix formation was almost terminated at this stage. The external form of the tooth crown was nearly completed and the root formation was initiated. The ameloblasts at the cusp site also started to become reduced enamel epithelium. The epithelial cells located at the cervical loop of the enamel organ in the developing tooth germ proliferated and extended toward the mesenchymal tissue, thus forming Hertwig's epithelial root sheath (HERS).

On P10, strong expression of FRG1 mRNA was observed in the ameloblasts secreting enamel matrix on the lateral sides of the tooth crown and at the presumptive buccal cusp site (Fig. 4a, black arrows). In addition, strong expression of FRG1 mRNA was also found in the


FRG1 mRNA
FRG1 protein
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odontoblasts in the presumptive cusp site (white arrow) and in preodontoblasts on the lateral sides of the tooth (white arrowhead) (Fig. 4a, b). A weak signal was found in the HERS (Fig. 4a, b, c, red arrow). The in situ FRG1 signal was detected in the stratum intermedium. The FRG1 protein expression pattern (Fig. 4d, f, g, h) was similar to that of the mRNA. FRG1 mRNA and protein were both strongly expressed in the odontoblasts and ameloblasts secreting enamel matrix, but their signal intensity became reduced in the enamel epithelium at the presumptive lingual cusp sites where ameloblasts became reduced enamel epithelium (Fig. 4g, yellow arrow). Weak FRG1 protein expression was seen in the HERS (Fig. 4h, red arrow).

Tooth eruption stage (P20)

The tooth root formation was almost completed by this stage, and the tooth crown was exposed at the surface of the oral mucosa. The ameloblasts/reduced enamel epithelium disappeared from the tooth at this stage. The expression of FRG1 mRNA was strongly expressed in the odontoblasts (Fig. 4i, j, white arrow). The FRG1 protein showed a similar expression pattern to the mRNA (Fig. 4l, n, o, white arrow).

Subcellular localization of the FRG1 protein in the mouse dental epithelial cells with or without BMP4 treatment

To investigate the subcellular localization of the FRG1 protein, immunocytochemistry using an anti-FRG1 antibody was performed using mDE6 cells. The FRG1 protein signal in the mouse tooth germ was detected in the cytoplasm in vivo (Figs. 2, 3, 4), while the FRG1 in mDE6 cells was expressed predominantly in the nucleus (Fig. 5a, d). BMP signaling has been reported to be critical for

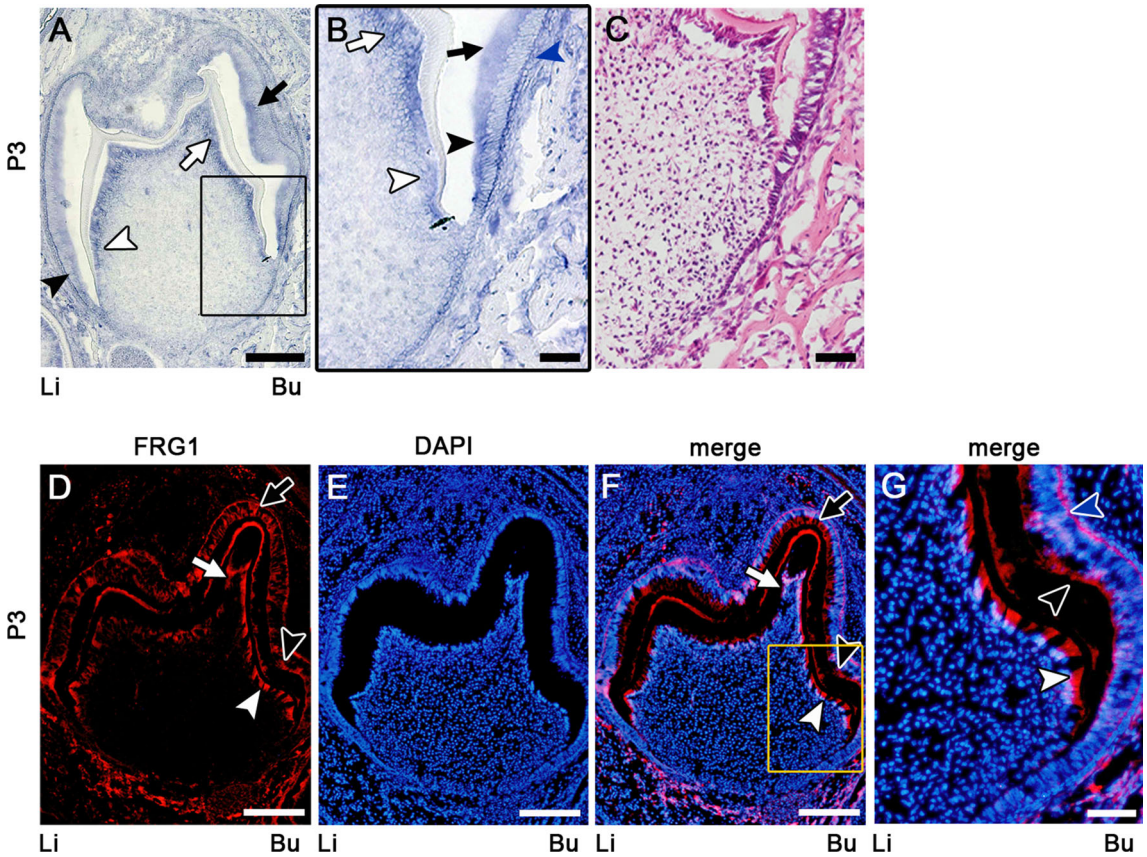

Fig. 3 The expression of FRG1 mRNA and protein in the tooth germ on P3**. a**, **b** The in situ signal for FRG1 was detected in the preameloblasts (*black arrowhead*), ameloblasts (*black arrow*), preodontoblasts (*white arrowhead*), odontoblasts (*white arrow*), and stratum intermedium (*blue arrowhead*). (**b**) shows a higher magnification image of the boxed area in (**a**). **c** The HE-stained image corresponding to (**b**). **d**, **f** The expression of FRG1 protein was observed in the ameloblasts (*black arrow*) and odontoblasts (*white arrow*) at the cusp sites and in the preameloblasts (*black arrowhead*) and preodontoblasts (*white arrowhead*) in the lateral sides of the tooth germ. **e** The nuclei were counterstained with DAPI. **g** The *boxed area* in (**f**) is shown at a higher magnification. The expression of FRG1 protein was also detected in the stratum intermedium (*blue arrowhead*). *Li*; lingual side, *Bu*; buccal side. *Scale bars*; 50 μm (**b**, **c**, **g**), 200 μm (**a**, **d–f**)

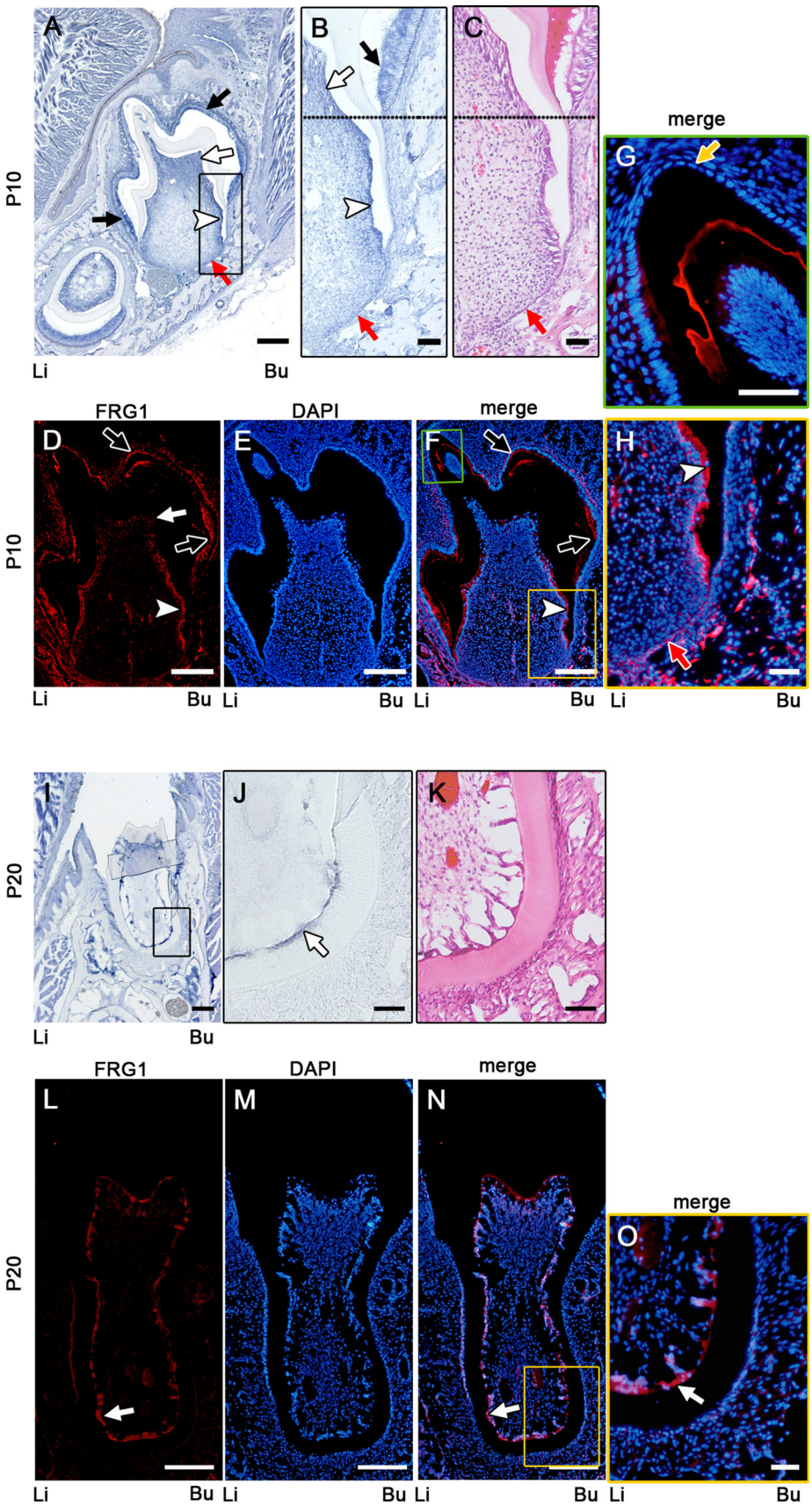

Fig. 4 The expression of FRG1 mRNA and protein in the tooth germ on P10 and P20**. a, b** On P10, FRG1 mRNA expression was observed in the ameloblasts (*black arrows*), odontoblasts (*white arrow*), preodontoblasts (*white arrowhead*) and HERS (*red arrow*). (**b**) shows a higher magnification image of the *boxed area* in (**a**). The *dotted line* indicated the border between the tooth crown and root. **c** The HE-stained section corresponding to (**b**). **d**, **f** The FRG1 protein was detected in the ameloblasts (*black arrows*), odontoblasts (*white arrow*) and preodontoblasts (*white arrowhead*) on P10. **g, h** The *green-* and *yellow-boxed areas* in (**f**) are respectively shown at a higher magnification. In (**g**), the FRG1 protein was not observed in the reduced enamel epithelium (*yellow arrow*). In (**h**), the FRG protein was detected in the preodontoblasts (*white arrowhead*) and HERS (*red arrow*). **i, j** On P20, strong expression of FRG1 mRNA was found in the odontoblasts (*white arrow*). (**j**) shows a higher magnification image of the *boxed area* in (**i**). **k** The HE-stained section corresponding to (**j**). **l**, **n, o** The FRG1 protein was detected in the odontoblasts (*white arrow*). (**o**) shows a higher magnification image of the *boxed area* in (**n**). **e**, **m** The nuclei were counterstained with DAPI. *Li*; lingual side, *Bu*; buccal side. *Scale bars*; 50 μm (**b–d**, **h**, **j**, **k**, **o**), 200 μm (**a**, **e**, **f**, **i**, **l–n**)

mammalian tooth development (Åberg et al. 1997; Thesleff 2003). The expression of BMP4, which is a member of the BMP family, was detected in the inner enamel epithelium on E18 and its expression pattern was similar to that of FRG1 (Fig. S2). Therefore, the FRG1 localization was immunocytochemically examined in mDE6 cells treated with BMP4. Intriguingly, the FRG1 protein was detected in the both nucleus and cytoplasm in the mDE6 cells treated with 5 ng/ml BMP4 (Fig. 5e, h). We next examined the protein expression level of FRG1 using the mDE6 cell extract fractionated to nuclear and cytoplasm by western blot analysis. Although there was no significant change in the total FRG1 protein expression level between untreated and BMP4-treated mDE6 cells (Fig. 6a), the cytoplasm FRG1 was significantly increased in the cells treated with BMP4 compared to mDE6 cells with no stimulation (Fig. 6b).

In addition, double-immunostaining for FRG1 and β-actin was performed using the mDE6 cells (Fig. 5b, f), because FRG1 has been reported to help stabilize the actin cytoskeleton (Sun et al. 2011). In the double-immunostained mDE6 cells treated with BMP4, the cytoplasmic FRG1 colocalized with β-actin (Fig. 5h).

Discussion

In this study, to unveil the potential role of FRG1 during odontogenesis in the mouse lower first molar, we performed in situ hybridization and immunohistochemistry. We also compared the subcellular localization of FRG1 in the BMP4-treated mDE6 cells to that of the untreated mDE6 cells. The in situ hybridization and immunohistochemistry studies showed a specific expression pattern of FRG1 during the tooth development. Of note, the subcellular translocation of FRG1 was induced by the BMP4 treatment.

At the initiation of tooth development, the expression of FRG1 mRNA and protein was observed in both the oral mucosal epithelium and mesenchymal cells on E10.5 and E12. Subsequently, the tooth morphology progresses toward the bud, cap and bell stages. At the bud and cap stages (E14–16), the expression of FRG1 mRNA and protein was detected in the enamel organ. A weak signal was also observed in the dental papilla. At the early bell stage (E18), a strong signal for FRG1 mRNA was detected in the inner enamel epithelium and the cervical loop, and a weak signal was found in the cells of the dental papilla facing the inner enamel epithelium. The FRG1 expression in the developing tooth germ, except for the enamel knot in the early stages (E14–18), corresponded with the localization of BrdU-positive cells, which represent cells with high proliferative activity, as observed in our previous studies (Shigemura et al. 1999). The FRG1 expression was also seen in the PEK (E15), which has been regarded as a signaling center regulating tooth crown morphogenesis (Jernvall et al. 1994; Vaahtokari et al. 1996). Therefore, FRG1 may be involved in the morphogenesis of the crown during tooth development.

At the differentiation of ameloblasts and odontoblasts, and the mineralization of enamel and dentin, which occurred on P0, a strong signal for FRG1 mRNA was detected in the preameloblasts and preodontoblasts at the lingual cusp site, where the predentin matrices first start forming. At the central groove region, where the formation of predentin was not observed, the expression of FRG1 mRNA and protein was not detected. Interestingly, on P1,

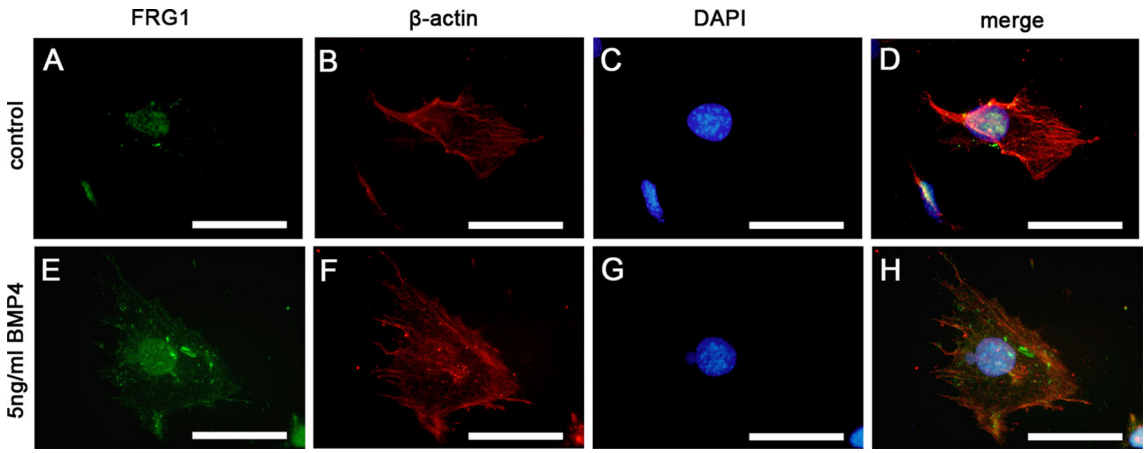

Fig. 5 The subcellular localization of the FRG1 expression in the dental epithelial cells. **a** The endogenous FRG1 (*green*) was localized predominantly in the nucleus of the mDE6 cells without BMP4 treatment. **e** The FRG1 protein in the mDE6 cells treated with 5 ng/ml BMP4 was localized in both the nucleus and cytoplasm. **b**, **f** The β-actin (*red*) was observed in the cytoplasm of the mDE6 cells. **d**, **h** Double-immunostaining showed a partial overlap of FRG1 (*green*) and β-actin (*red*). **c**, **g** The nucleus was stained with DAPI. *Scale bars*; 50 μm

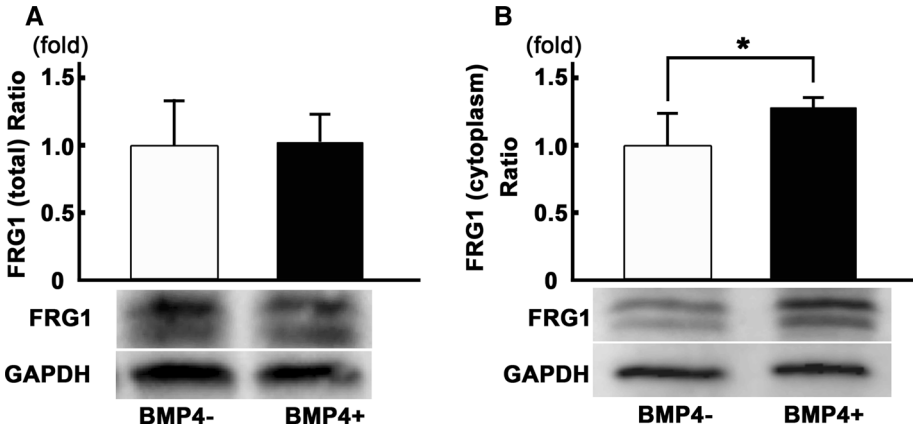

Fig. 6 The translocation of FRG1 by BMP4. **a** There was no significant difference in the total protein expression between untreated and BMP4-treated mDE6 cells. **b** The expression level of cytoplasm FRG1 was significantly increased in mDE6 cells stimulated by BMP4 compared to mDE6 cells with no stimulation. The data is mean ± SD from triplicate samples. $*P < 0.01$ versus BMP4- by a one-way ANOVA with the Tukey–Kramer comparison test

signals for the FRG1 mRNA and protein were observed in the preameloblasts and preodontoblasts at the central groove region after the initiation of the matrix formation. The FRG1 mRNA and protein also continued to be expressed in the ameloblasts and odontoblasts at the lingual cusp site where the matrix formation was observed. On P3, the matrix was formed at the lateral sides of the tooth crown, and the expression of FRG1 was detected in the preameloblasts and preodontoblasts at this site, and in the ameloblasts and odontoblasts at the occlusal site of the tooth crown. Moreover, at the root formation stage (P10, 20), FRG1 was expressed in the preodontoblasts and odontoblasts at the tooth root, while FRG1 was not found in the reduced enamel epithelium in the lingual cusp site on P10. Thus, FRG1 was expressed in the preodontoblasts, odontoblasts, preameloblasts and ameloblasts corresponding to the stage of the matrix formation. These results suggest that FRG1 is involved in the differentiation of ameloblasts and odontoblasts. In addition, FRG1 may be associated with the secretion and formation of enamel and dentin matrices.

In this study, the FRG1 protein in the mouse tooth germ was localized to the cytoplasm in vivo, while nuclear FRG1 was detected in the mDE6 cells in vitro. Endogenous FRG1 is a dynamic nuclear–cytoplasmic shuttling protein (Sun et al. 2011). The nuclear FRG1 is associated with multiple aspects of RNA biogenesis, including RNA splicing and mRNA transport (Sun et al. 2011). On the other hand, cytoplasmic FRG1 may have a structural role in stabilizing the actin cytoskeleton (Sun et al. 2011). FRG1 may play different roles depending on its subcellular localization. It has been reported that BMP4 expression was detected in the ameloblasts and odontoblasts during the differentiation of these cells, and during the deposition of enamel and dentin matrices (Åberg et al. 1997; Thesleff 2003). The expression pattern of FRG1 during tooth development in this study was similar to that of BMP4. Thus, immunocytochemical studies were performed using mDE6 cells treated with BMP4. The subcellular FRG1 in the mDE6 cells treated with BMP4 appeared to be partially translocated from the nucleus to the cytoplasm. Furthermore, western blot analysis showed that cytoplasmic protein was significantly increased in the mDE6 cells treated with BMP4, although no marked changes were observed in the total FRG1 protein. These results suggested that FRG1 was translocated from the nucleus to the cytoplasm by BMP4 treatment. In addition, the cytoplasmic FRG1 displayed an overlap with β-actin. This finding was identical to that in a previous study (Sun et al. 2011). Furthermore, the FRG1 expression was observed to be located in the cytoplasm of the ameloblasts and odontoblasts which formed the enamel and dentin matrix, respectively. The actin cytoskeleton has been reported to be important for the differentiation of ameloblasts and odontoblasts via the regulation of the amelogenin and DSPP expression (Biz et al. 2010). Thus, the present findings suggest that the cytoplasmic FRG1 may also be involved in the tooth germ development, including the generation of enamel and dentin matrix through β-actin formation.

In the present study, we demonstrated the stage-specific temporal and spatial expression patterns of FRG1 mRNA and protein during tooth development in mice. Based on the expression pattern during odontogenesis in the mouse, FRG1 appears to be related to the morphogenesis of the tooth germ, the differentiation of ameloblasts and odontoblasts and matrix formation. Furthermore, FRG1 was localized in the cytoplasm of ameloblasts and odontoblasts in vivo, while its expression was translocated into the

cytoplasm and colocalized with β-actin in the mouse dental epithelial cells treated with BMP4 in vitro. Because the mechanism underlying the translocation of FRG1 by BMP4 treatment remains unknown, further studies are necessary to understand the interaction between FRG1 and BMP4 and the biological function(s) of FRG1 during tooth germ development. However, the present study demonstrated the expression pattern of FRG1 in the developing tooth germ, and thus suggests the possible role of FRG1 in tooth germ morphogenesis.

Acknowledgments The authors disclosed receipt of the following financial support for the research described in this article: Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Nos. 25462862 (to K.N.), 15H05023 (to N.W.) and 26670826 (to N.W.).

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