Tooth Morphogenesis and FGF4 Expression During Development of Molar Tooth in Three Muroid Rodents: *Calomyscus elburzensis* (Calomyscidae), *Mesocricetus auratus* (Cricetidae) and *Mus musculus* (Muridae)

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ABSTRACT

To date, no studies have examined the tooth formation during developmental stages of brush-tailed mice (Calomyscidae) and true hamsters (Cricetidae). Herein, we compared the timing of tooth morphogenesis and FGF4 expression pattern during development of the first lower molar in Goodwin's brush-tailed mouse, *Calomyscus elburzensis* with two other muroid rodents; the house mouse, *Mus musculus* (Muridae), model organism for tooth morphogenesis, and the golden hamster, *Mesocricetus auratus* which shares great similarities in cusp pattern with brush-tailed mice. All three species were bred in captivity and developing embryos were isolated at different embryonic days (E). Histological evaluation of lower molars was performed and spatiotemporal pattern of FGF4 expression was determined by immunohistochemistry. Results indicated that morphogenesis of the tooth cusps starts at the beginning of the cap stage of the first lower molar (E14 in house mouse, about E11.5 in golden hamster and E22 in Goodwin's brush-tailed mouse). During the cap to bell stage (E15 in house mouse, E12 in golden hamster and at about E24 in Goodwin's brush-tailed mouse), a decrease in the expression of FGF4 was observed in the mesenchyme, except for the cusp tips. According to our

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observations, the developmental process of the first lower molar formation in Goodwin's brush-tailed mouse began much later as compared with the other two species. Despite the differences in the temporal pattern of molar development between these three members of the same superfamily (Muroidea), the correlation in the expression of FGF4 with specific stages of tooth morphogenesis supported its regulatory function. Anat Rec, 300:2138–2149, 2017.

Key words: tooth morphogenesis; molar cusp pattern; histology; immunohistochemistry; FGF4 expression; *Calomyscus*; *Mesocricetus*

Tooth emergence can be considered as a key innovation in vertebrate evolution and the variability of the shape of the tooth crown has played a major role in mammalian radiation (Harjunmaa, 2012). Because tooth morphology varies considerably among mammal species, it provides an array of characters that can be used for identification of recent, archaeological, and fossil materials. Fragments of the bony skeleton rarely provide precise information regarding identification of the source, but an isolated tooth can often be used to identify a particular species. Studies of mammalian evolution frequently use dental characters which are particularly crucial for inferring phylogenetic relationships of fossil taxa, of which teeth are often the only recovered part. Hence, comparative dental anatomy is an important subject for mammalogists, paleontologists, and archaeologists (Luo et al., 2001; Woodburne et al., 2003; Hillson, 2005). Teeth are a vital part of an animal's interaction with its environment and thus directly affect fitness. All in all, teeth provide a unique research focus for studies related to evolution, development and functional morphology (Harjunmaa, 2012).

All teeth, regardless of their shape or morphology, are formed by similar tissues and experience similar developmental stages (Keränen et al., 1999). Because of this similarity in development, teeth are considered as homologous series and regulation of their morphogenesis and cell differentiation should be conserved among different species. Therefore, the species-specific differences in the numbers (and locations) of tooth germs could be traced via the assessment of local modifications in the expression patterns of genes involved in the initiation of tooth germ development (Keränen et al., 1998, 1999). In other words, determination of tooth region, and subsequently tooth type and tooth cusps depends on the signaling molecules that regulate growth and differentiation of the tooth tissue. In this regard, the signaling families of bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and hedgehog (HH) are particularly important (Jernvall and Thesleff, 2000).

Transient signaling centers in the epithelium corresponding to the initiation of tooth buds, tooth crowns and individual cusps, punctuate the advancing tooth morphogenesis. The enamel knot which is a transient epithelial structure, contributes to directing the folding and the growth of the dental epithelium (Jernvall and Thesleff, 2000). The primary enamel knot is an organizing center which induces the formation of secondary enamel knots. Spatiotemporal induction of the secondary enamel knots and the regulatory molecules, including BMP2, BMP4 and BMP7, as well as FGF4, which are expressed in the secondary enamel knots of the future cusps, contribute to differences in tooth morphology among various species (Keränen et al., 1998; Jernvall and Thesleff, 2000).

To date, experimental research on tooth development among rodents is based very largely on the teeth of murine rodents. Pioneering work on the house mouse tooth germ cultures gave detailed information about histological, structural and gene expression patterns during its prenatal development (Thesleff et al., 1988, 1995; Maas and Bei, 1997; Thesleff and Sharpe, 1997; Keränen et al., 1998, 1999; Nadiri et al., 2004). In the mouse embryo, the morphological development of molar tooth commences at E11, with thickening of the oral epithelium which is considered as dental lamina. At E12, the dental lamina invaginates into the underlying mesenchyme of the first branchial arch, then proliferates and condenses, forming the early and late bud stages at a bit after E12 and E13, respectively (Tucker and Sharpe, 2004; Nanci, 2008). At late bud stage, FGF4 is observed in the basement membrane, epithelium and mesenchyme (Nadiri et al., 2004). Subsequently, the epithelium becomes convoluted, forming the cap and bell stages at E14 and E16, respectively (Tucker and Sharpe, 2004; Nanci, 2008). From the bud to the bell stage, FGF4 is mainly localized in the basement membrane however, at the cap stage (E14 and E15) FGF4 staining is more intense in the enamel organ than the mesenchyme (Nadiri et al., 2004). Consequently, at about E17, the epithelial-mesenchymal interactions culminate in the differentiation of the epithelium into enamel-secreting ameloblasts and differentiation of the mesenchyme into dentin-secreting odontoblasts (Tucker and Sharpe, 2004; Nanci, 2008). At this time, the staining for FGF4 is very strong in the inner and outer dental epithelia and weak staining is detected at the tips of the cusps. At E19 tooth shows the late bell stage and FGF4 expression is significantly decreased (Nadiri et al., 2004).

Studies have shown the regulatory functions of *Fgf4* gene in various steps of tooth development in house mouse including early development of tooth germ, morphogenesis of tooth cusps, activating early formation of cusps and their growth, and also inducing cell proliferation. FGF4 expression is used as an enamel knot marker in the onset

of species-specific tooth cusp patterns and developmental process of tooth crown (Kettunen and Thesleff, 1998; Kernen et al., 1998, 1999; Jernvall and Thesleff, 2000; Nadiri et al., 2004). Keränen et al. (1998) noted that *Fgf4* has closer spatial correlation to tooth morphology and is more relevant to patterning and evolution of species-specific tooth shapes than other genes, such as *Msx1*, which has roles in normal tooth development.

In this study morphogenesis and FGF4 expression pattern in the first lower molar were compared among three species of rodents; the house mouse (*Mus musculus* Linnaeus, 1758), the golden hamster (*Mesocricetus auratus* Waterhouse, 1839) and Goodwin's brush-tailed mouse (*Calomyscus elburzensis* Goodwin, 1938). The house mouse of the family Muridae is considered as a model organism for mammalian odontogenesis. The golden hamster is a member of a closely related family (Cricetidae) which has a much simpler molar cusp pattern (Fig. 1). These two rodents are compared with Goodwin's brush-tailed mouse with a molar cusp pattern very similar to that found in golden hamster (Fig. 1) but placed in the family Calomyscidae which has been found to be the sole member of a clade basal to the radiations of the Muridae and Cricetidae (Michaux et al., 2001; Steppan et al., 2004).

MATERIALS AND METHODS

Animals

Ten male and 10 female Soori albino house mice (*Mus musculus domesticus*), ~4-weeks old and averaging 26 ± 0.6 g in weight, and similar numbers of short hair golden hamsters (*Mesocricetus auratus*), ~3-weeks old and averaging 29 ± 0.4 g, were purchased from Razi Vaccine and Serum Research Institute in Mashhad and a local pet shop, respectively. Twelve adult male and 15 adult female Goodwin's brush-tailed mice (*Calomyscus elburzensis*) with average weight of 19 ± 0.9 g were live trapped in Mashhad (Hamidi et al., 2015). Mating were accomplished by pairing a single male and a single female in separate cages in an animal house (temperature of 24°C −28°C, humidity of 25%–35%, and with a 12 h light and dark cycle) and the day following the overnight mating was considered embryonic day 0 (E0). Pregnancy was obtained for ten house mice, seven golden hamsters and nine Goodwin's brush-tailed mice. Female house mice were euthanized and their embryos isolated at E10, E12, E13, E14, E15, E16 and E17. Female golden hamster's embryos were harvested at E9, E10, and E13 because of their known shorter gestation period (Soderwall et al., 1960), whereas brush-tailed mice embryos were harvested at E13, E14, E15, E17, E22, and E25 because of their known longer gestation period (Volf and Volf, 2003). Newborn pups (postnatal day 0: PN0) from each species were also included in further studies. Experimental procedures were performed in accordance with the "care and use of animals" protocols approved by the ethics committee of Ferdowsi University of Mashhad, Iran.

Histological Evaluation

The craniofacial region of euthanized embryos and newborn pups was dissected and fixed in 10% neutral buffered formalin for at least 24 h. The samples were dehydrated in ethanol and embedded in 50°C −54°C paraffin. Serial sagittal and transverse sections, 7 μm in thickness, were made by a rotary microtome (Leica Leitz; 1512; Germany), treated with xylene and ethanol, and stained with hematoxylin. Cells were identified by their oval or round nuclei and their blue-colored cytoplasmic granules in hematoxylin stained sections. Imaging was performed using a light microscope (Olympus BH2, Japan) connected to a DP71 digital camera (Japan). The stage of the tooth development for each represented embryonic day and for the first postnatal day in all three species was determined based on histological changes.

Immunohistochemical Procedure

The expression of FGF4 was examined in a series of sagittal and transverse sections of the craniofacial regions. The sections were prepared on poly-L-lysine coated glass slides following a modified protocol of Nadiri et al. (2004). To detect FGF4 masked by formalin fixation and paraffin embedding, tissue sections were pretreated by microwaving in sodium citrate (pH 6) at 95–100°C. After rinsing with tris-buffered saline (TBS, pH 6–7), the slides were incubated for 15 min in 3% H_2O_2 in TBS to neutralize endogenous peroxidase activity. Nonspecific binding sites were blocked by placing slides in 4% bovine serum albumin (BSA) in TBS for 45 min. Slides were incubated at least overnight in the presence of the primary antibody, anti-FGF4 (Santa Cruz Biotechnology, Cat. No. sc-1361) (dilution 1:300 in 4% BSA), washed three times with TBS, and incubated for about 2 h in rabbit anti-goat IgG H&L (HRP) secondary antibody (Abcam, Cat. No. Ab6741) (dilution 1:150 in 4% BSA). Following three rinses in TBS, visualization of FGF4 was obtained by incubating sections with DAB (3,3′-diaminobenzidine tablets) solution (pH 7.2). After dehydration and final deparaffinization, each slide was stained with hematoxylin (modified from Nadiri et al., 2004). Developing house mouse first lower molar sections served as positive controls for the primary antibody at different stages (Nadiri et al., 2004) and omission of the primary antibody was considered as negative control.

RESULTS

In this study, the following embryonic and postnatal time points were inspected in details: E13 and PN0 (all three species), E9 (golden hamster), E10 (house mouse and golden hamster), E12 and E16 (house mouse), E14, E15, and E17 (house mouse and Goodwin's brush-tailed mouse), and E22 and E25 (Goodwin's brush-tailed mouse) (Fig. 2). It is worth noting that since there was no background information on prenatal development in either golden hamsters or brush-tailed mice, we initially considered four main embryonic days (E13, 14, 15, and 17) based on previous data on the house mouse (e.g., Keränen et al., 1998; Nadiri et al., 2004). Based on our previous observations (unpublished data), the mean gestation period in golden hamster was found to be 15.6 ± 0.8 days, which was about 5 days shorter than that observed for house mouse (20.8 ± 0.8). Our attempts for providing embryos at E14 for golden hamster were unsuccessful partly due to close proximity of this day to

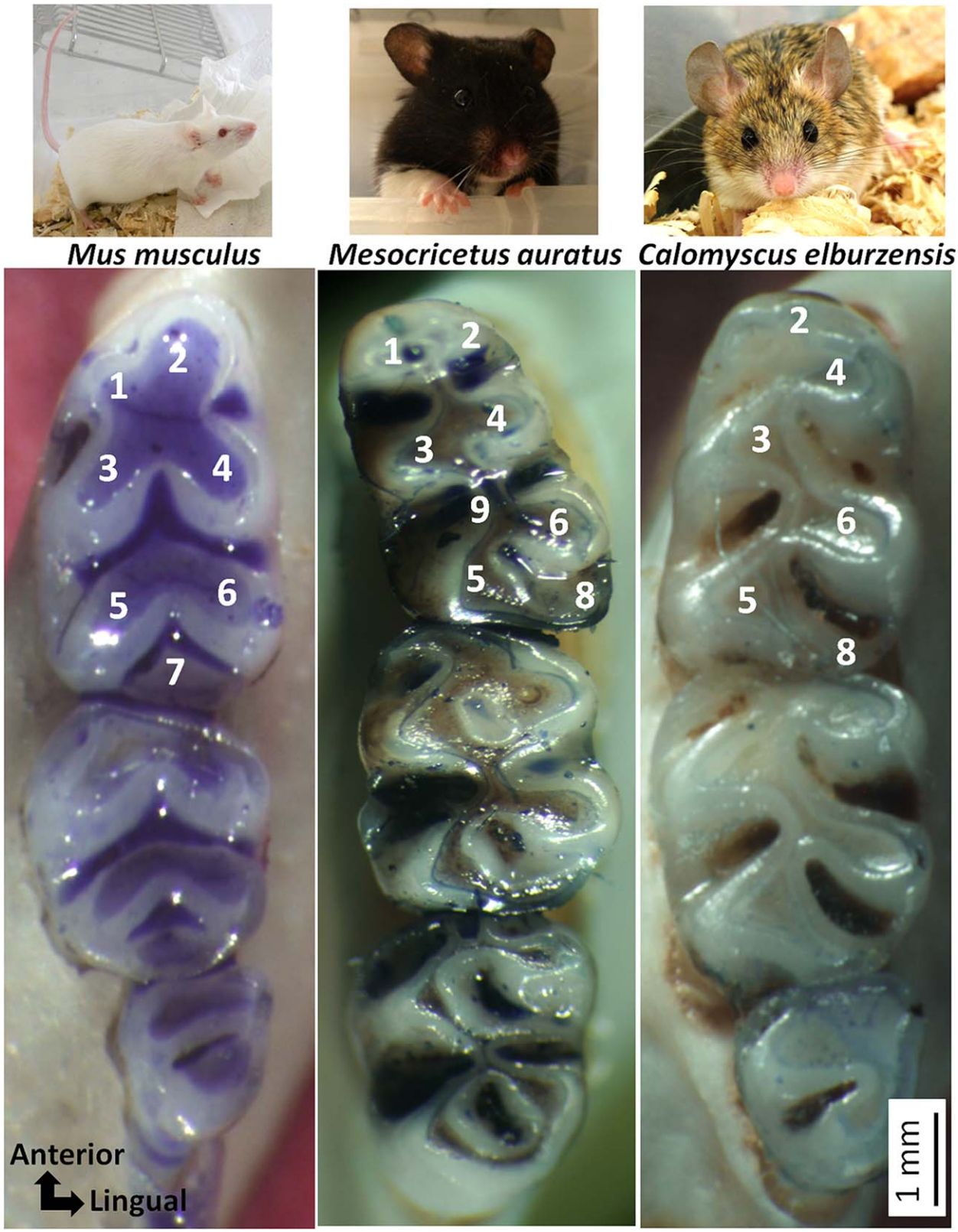

Fig. 1. Lower left tooth row of three examined muroid rodents: house mouse (*Mus musculus*), golden hamster (*Mesocricetus auratus*) and Goodwin's brush-tailed mouse (*Calomyscus elburzensis*). Teeth have been stained blue in the first two species to aid in visualizing the tooth morphology. 1: anterolabial conulid, 2: anterolingual conulid, 3: protoconid, 4: metaconid, 5: hypoconid, 6: entoconid, 7: posterior cingulum, 8: posterostylid, 9: longitudinal crest (cusp nomenclature based on modification after Wood and Wilson, 1936).

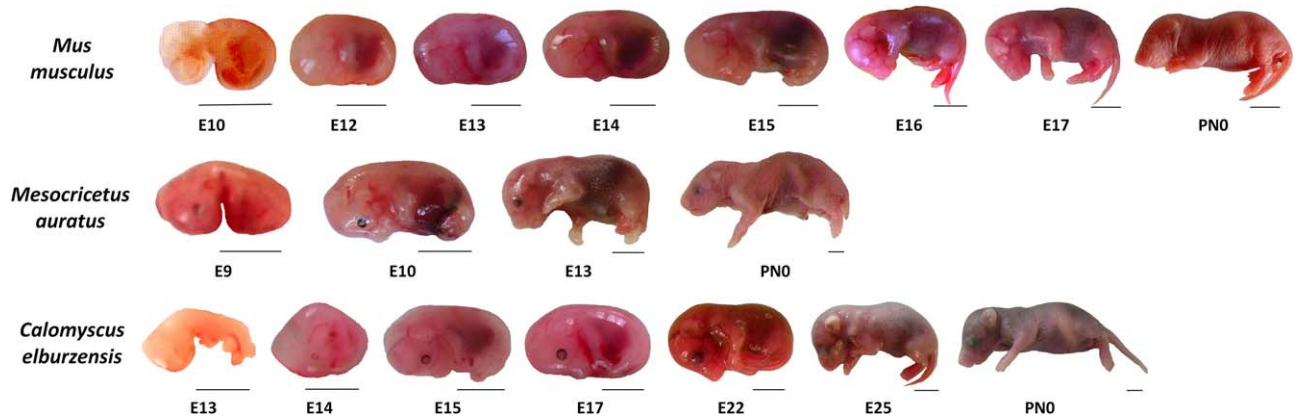

Fig. 2. Morphological comparison at different embryonic days (E) and first postnatal day (PN0) in house mouse (*Mus musculus*), golden hamster (*Mesocricetus auratus*) and Goodwin's brush-tailed mouse (*Calomyscus elburzensis*). Scale bars indicate 5 mm.

the parturition time in this species. Selecting additional embryonic days for completing the series of developmental stages in each species was based on progressive histological observations. For example, during this study it became clear that before E10, E9, and E13 in house mouse, golden hamster and Goodwin's brush-tailed mouse, respectively, the craniofacial regions were not sufficiently differentiated and these data were excluded from our study. Furthermore, E22 and E25 for Goodwin's brush-tailed mouse were added based on progressive histological observations during the study and the differences observed in the timing of embryo development among Goodwin's brush-tailed mouse and the other two examined species. In Goodwin's brush-tailed mouse pregnancy duration averaged 31.5 ± 2.1 days (unpublished data).

Morphogenesis of the First Lower Molar

Histological comparisons revealed that the first lower molars in all three species had great similarities in their general developmental stages, consisting of the thickening of the dental lamina, epithelial budding and subsequent morphogenesis. Each developmental stage was defined based on general morphology of the tooth germ. Despite the differences in developmental timing, the gross histology of the tissues appeared similar among the three species (Table 1).

By E10, house mouse embryo had developed enough to be recognized at least as a mammal embryo (Fig. 2) and all tissues including the craniofacial region showed very weak differentiation (Fig. 3A). Golden hamster and Goodwin's brush-tailed mouse demonstrated this state of development at about E9 and E13, respectively (Figs. 2, 4A, and 5A). At E12, a very weak localized thickening (placode) within the oral epithelium, initiating the first lower molar development (early bud stage) was observed in house mouse (Fig. 3B). This occurred at E10 in golden hamster (Fig. 4B) and E14 in Goodwin's brush-tailed mouse (Fig. 5B). At E13 in house mouse and about E11 in golden hamster, the early buds changed appearance to mature buds (late bud stage) however; this was not observed until E17 in Goodwin's brush-tailed mouse. At the late bud stage, primary enamel knot cells formed as a histologically distinct epithelial mass at the tip of the epithelial bud (data not shown). The cap stage occurred at E14 in house mouse (Fig. 3C), a bit after E11 in golden hamster and at E22 in Goodwin's brush-tailed mouse (Fig. 5C). The bell stage was observed between E16 and E19 in house mouse (Fig. 3D,E) and between E12.5 and E13.5 in golden hamster (Fig. 4C). In Goodwin's brush-tailed mouse the bell stage started at E25 (Fig. 5D,E) and continued after birth. During the E25–31 (early bell stage) in this species, the adjacent epithelial and mesenchymal cells of the crown began to differentiate into ameloblasts (Fig. 5E) and odontoblasts, respectively. At PN0 in both house mouse (Fig. 3F,G) and golden hamster (Fig. 4D,E) a definite layer of dentin was formed. Although the dentin and enamel matrix were formed in newborn pups (PN0) of the golden hamster, the matrix continued its development after birth in the house mouse. In Goodwin's brush-tailed mouse, at PN0, tooth germ development of the first lower molar was at intermediate bell stage, with a definite layer of predentin (Fig. 5F–H).

FGF4 Expression Pattern during First Lower Molar Morphogenesis

In this study, we aimed to compare the interspecific comparisons of spatiotemporal expression patterns of FGF4 in developing molar teeth in different species at similar stages of development (our comparison criteria), rather than similar ages of embryos. FGF4 positive cells were observed in groups or singly dispersed within the tooth tissue in all investigated sections. The developing house mouse first lower molar tissue for which the primary antibody was omitted was used as a negative control (Fig. 3E). Variation in FGF4 expression in tissues was inferred from the intensity of FGF4 immunostaining, which varied from blue (no FGF4 expression) to lighter staining or completely brownish (weaker and intense FGF4 staining, respectively). Generally weaker immunostaining for FGF4 was observed in the lower

TABLE 1. Histomorphological comparison of first lower molar shape development in house mouse (*Mus musculus*), golden hamster (*Mesocricetus auratus*), and Goodwin's brush-tailed mouse (*Calomyscus elburzensis*), three muroid rodents

	Embryonic (E)/postnatal (PN) days		
The developmental event	*Mus musculus*	*Mesocricetus auratus*	*Calomyscus elburzensis*
Very weak differentiation in craniofacial tissues	E10	E9	E13
Early bud stage; emerging of a thickening within the epithelium	E12	E10	E14
Late bud stage; formation of primary enamel knot	E13	around E11	E17
Cap stage; developing of primary enamel knot, initiation of crown and cusp formation	E14	at probably E11.5	E22
Cap/bell stage; initiation of secondary enamel knots formation	E15	E12	around E24
Early bell stage; probable apoptosis of secondary enamel knots, initiation phase in emerging of ameloblasts and odontoblasts, appearing of cusps in their species-specific positions	E16	E12.5	E25
Intermediate bell stage; establishment of tooth crown base, emerging of ameloblasts and odontoblasts	E17	E13	PN0
Apposition/calcification; forming definite layer of enamel and dentin	PN0 and after that	PN0	after PN0

molars of golden hamster compared with the other two species (Fig. 4 vs. Figs. 3 and 5) which may be related to differences in the affinity of the immunostaining to FGF4 in this species. Immunolocalization of FGF4 in the developing first lower molars was compared among house mouse, golden hamster and Goodwin's brush-tailed mouse (Figs. 3–5, respectively, and also Table 2).

Bud to Cap Transition

In house mouse, FGF4 could be detected in the epithelium, mesenchyme and basement membrane at E13; however its expression was higher in the latter. By around E11 in golden hamster and by E17 in Goodwin's brush-tailed mouse intense staining in both oral epithelium and basement membrane were observed (data not shown). At the late bud stage, the transition to cap stage begins and the primary enamel knot at the tip of the epithelium is formed (Jernvall et al., 1994). In house mouse, FGF4 was distributed mainly in the oral epithelium at E14. Conversely, the staining was weak in underlying cells including mesenchyme, inner and outer dental epithelia (Fig. 3C). The transition to cap stage may occur at about E11.5 in golden hamster and at E22 to E25 in Goodwin's brush-tailed mouse (Fig. 5C). The relative amount of FGF4 decreased between E14 and E15 in house mouse. During this period staining for FGF4 was mainly observed in association with the inner and outer dental epithelia, while the mesenchyme in the central part of the tooth showed a decrease for FGF4 staining (data not shown). After E15 in house mouse, E12.5 in golden hamster and E25 in Goodwin's brush-tailed mouse (Fig. 5D) the weak staining for FGF4 in the dental mesenchyme progressively disappeared.

Cap to Bell Transition

In house mouse, the first cusps morphologically appeared at E16 (early bell stage) with the formation of secondary enamel knots. Secondary enamel knots are clusters of dental epithelial cells which are seen at the tip of the forming cusps (Jernvall et al., 1994). From E15 to E17 in house mouse, staining for FGF4 was mainly observed in association with inner and outer dental epithelia (Fig. 3D) and was decreased in the mesenchyme. This stage of cusp development was observed between E11.5 and E13 in golden hamster (Fig. 4C) and between E25 and PN0 in Goodwin's brush-tailed mouse (Fig. 5F–H).

Late Bell Stage and Cytodifferentiation

From E17 to E19 in house mouse, the relative amount of FGF4 decreased and the immunostaining for FGF4 was very weak in all dental tissues by E19 (Nadiri et al., 2004). This loss of FGF4 expression occurred at E13 to PN0 (Fig. 4D,E) in golden hamster and after PN0 in Goodwin's brush-tailed mouse.

DISCUSSION

Our observations for first lower molar development in house mouse were congruent with previous published data (Nadiri et al., 2004) (Table 2). We found that in Goodwin's brush-tailed mouse, the developmental process of first lower molar formation (Table 1) begins ~2 days later in gestation as compared with the house mouse (at E14 vs. E12) and some 4 days later compared with the golden hamster (E10). Furthermore, differences in the timing of morphogenesis of lower molar tooth were followed, with the beginning of the cap stage being

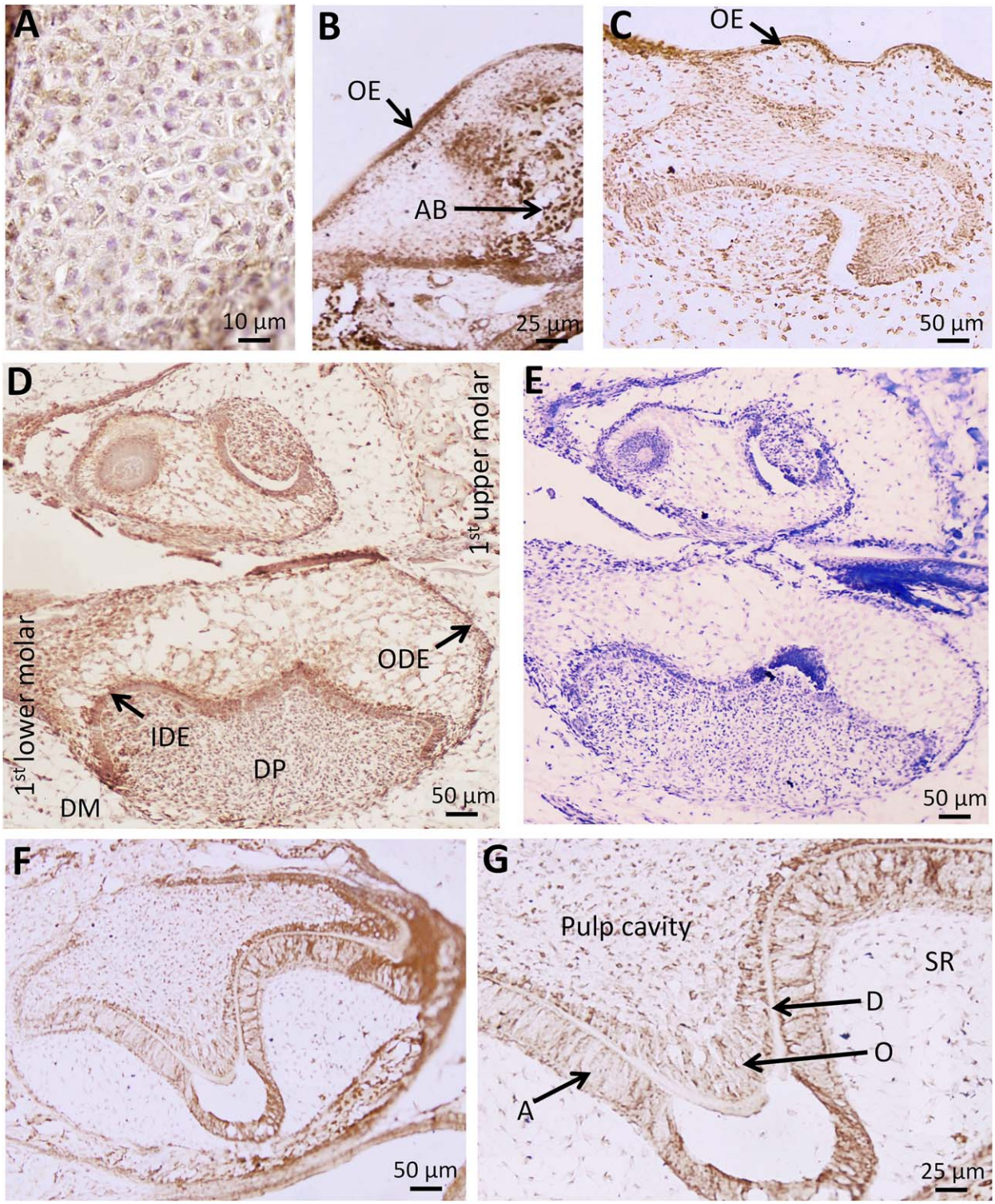

Fig. 3. Morphogenesis of the first lower molar shape and immunolocalization of the signaling molecule FGF4 in the house mouse. At E10 (A), no obvious differentiation in the craniofacial tissues was distinguished and FGF4 expression was observed in some cells throughout the tissue. At E12 (B), very weak thickening within the oral epithelium with intense FGF4 expression was observed. FGF4 expression in the underlying mesenchyme was weak however; the forming alveolar bone was intensely stained. At E14 (C), tooth showed the early cap stage and intense staining for FGF4 was observed in the oral epithelium. Conversely, the staining was weak in underlying cells including mesenchyme, inner and outer dental epithelia. At E17 (D), the tooth germ was in the intermediate bell stage and there was intense immunostaining for FGF4 in dental papilla, inner and outer dental epithelia. Conversely, the staining was weak in dental mesenchyme. E is the negative control (in which the primary antibody was omitted) in a similar position to section D and only blue staining with hematoxylin was observed. At PN0 (F, G), tooth was in the apposition stage; staining for FGF4 was mainly observed in odontoblasts and ameloblasts, definite layer of dentin was formed (G) and complete formation of dentin and enamel matrix continued after birth. E and PN indicate embryonic and postnatal days, respectively. A: ameloblasts, AB: alveolar bone, D: dentin, DM: dental mesenchyme, DP: dental papilla, IDE: inner dental epithelium, O: odontoblasts, ODE: outer dental epithelium, OE: oral epithelium, SR: stellate reticulum.

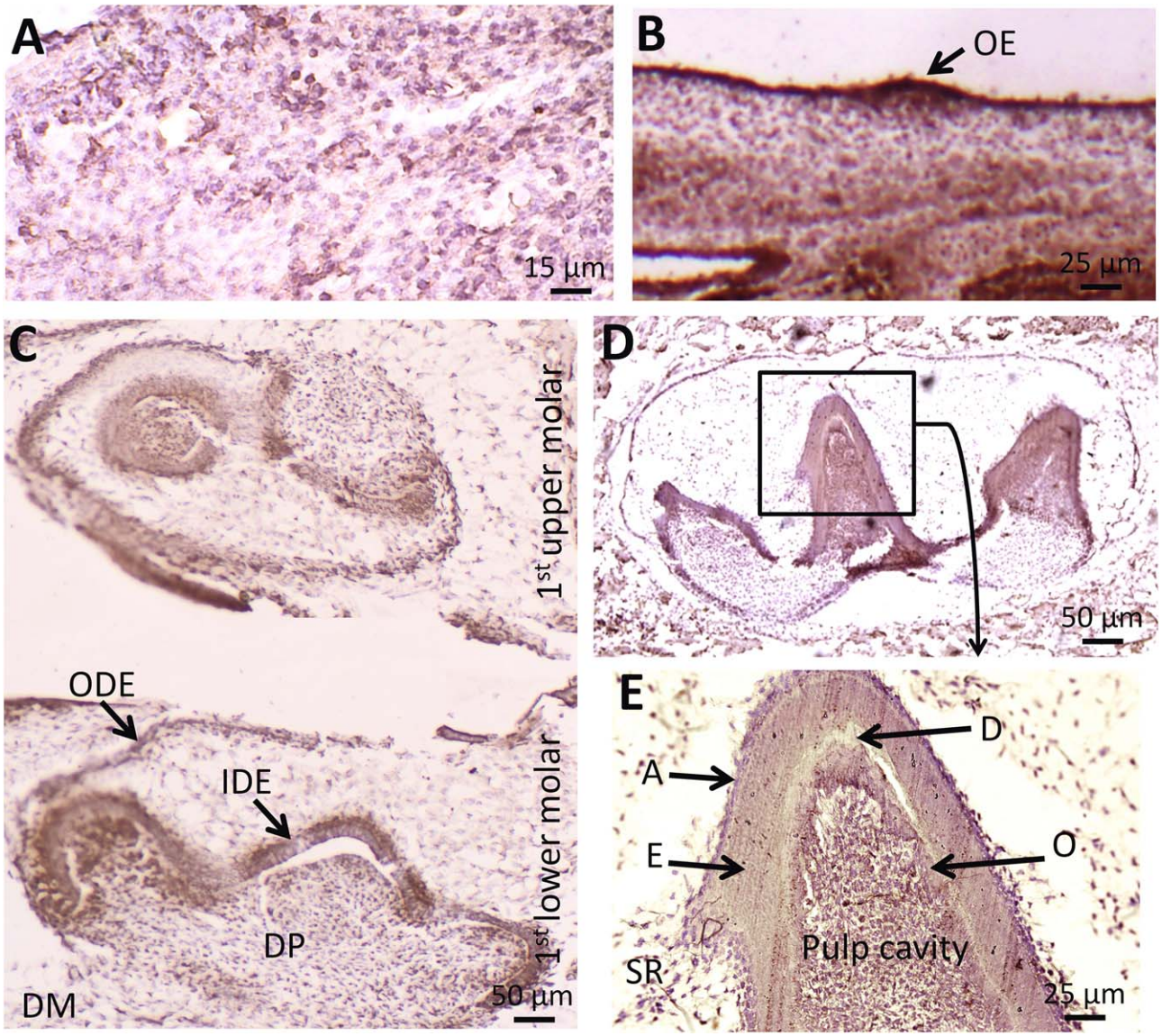

Fig. 4. Morphogenesis of the first lower molar shape and immunolocalization of the signaling molecule FGF4 in the golden hamster. At E9 (A), craniofacial tissues were not well developed and FGF4 expression in the whole tissue was observed. At E10 (B), very weak thickening within the oral epithelium was detected. FGF4 expression in the oral epithelium was intense as compared with the underlying mesenchyme. At E13 (C), tooth showed the intermediate bell stage and intense staining for FGF4 was observed in the dental papilla, inner and outer dental epithelia. Conversely, the staining was weak in dental mesenchyme. At PN0 (D, E), tooth showed the apposition/calcification stage; definite layer of dentin was formed and complete formation of enamel matrix was observed. There was weak immunostaining for FGF4 in all dental tissues except the pulp cavity at the tip of cusps. E and PN indicate embryonic and postnatal days, respectively. A: ameloblasts, D: dentin, DM: dental mesenchyme, DP: dental papilla, E: enamel, IDE: inner dental epithelium, O: odontoblasts, ODE: outer dental epithelium, OE: oral epithelium, SR: stellate reticulum.

observed at E14 in house mouse, at about E11.5 in golden hamster and at E22 in Goodwin's brush-tailed mouse.

In congruent with previous literature, we observed that during the bud stage the cervical loop was formed and the separation of inner dental epithelium and outer dental epithelium by the stellate reticulum occurred due to the histogenesis of the dental epithelium. Furthermore, primary enamel knot appeared in the central part of the enamel organ too. At the cap stage, FGF4 was detected in the oral epithelium (Figs. 3C and 5C). FGF4 has a role in stimulating cell proliferation in the epithelium and mesenchyme and also inhibits apoptosis in the dental mesenchyme (Vaahtokari et al., 1996; Nadiri et al., 2004). Hence, during the cap to bell stage transition, the elongation of cervical loop was observed which surrounded the dental mesenchyme. Furthermore, similar to previous studies the formation of secondary

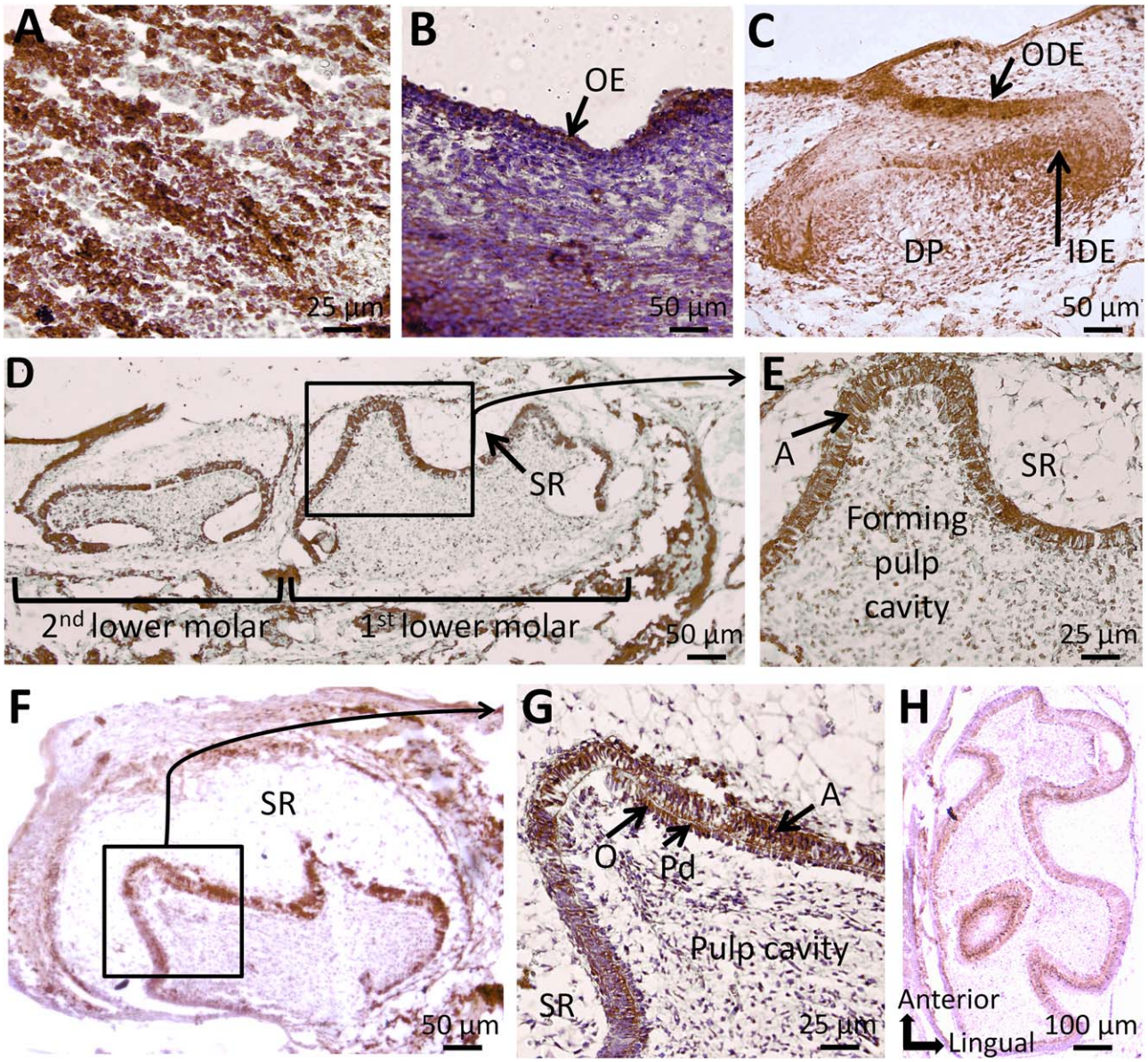

Fig. 5. Morphogenesis of the first lower molar and immunolocalization of the signaling molecule FGF4 in Goodwin's brush-tailed mouse. At E13 (A), craniofacial tissues showed no obvious differentiation and intense expression of FGF4 was observed throughout the section. At E14 (B), thickening within the oral epithelium and invagination into the underlying mesenchyme was observed and immunostaining for FGF4 in the oral epithelium and underlying mesenchyme was intense. At E22 (C), the tooth germ was at early cap stage. FGF4 intense staining was observed for oral epithelium, inner and outer dental epithelia. At E25 (D), the tooth germ of the first lower molar was in the early bell stage, but the second lower molar showed the late cap stage. Odontoblasts were not completely formed in the first lower molar (E). FGF4 intense staining was observed for the forming ameloblasts of the first lower molar (E). At PN0, tooth showed the intermediate bell stage (F). Complete formation of ameloblasts and odontoblasts occurred and definite layer of predentin was formed (G). Secretion of enamel matrix and dentin occurred after birth. Intense immunostaining for FGF4 was observed in ameloblasts and odontoblasts (G). In H, the transverse section of first lower left molar at PN0 is shown. Intense immunostaining for FGF4 was detected in outer layers of tooth (ameloblasts and odontoblasts). E and PN indicate embryonic and postnatal days, respectively. A: ameloblasts, DP: dental papilla, IDE: inner dental epithelium, O: odontoblasts, ODE: outer dental epithelium, OE: oral epithelium, Pd: predentin, SR: stellate reticulum.

enamel knots as a result of segregation of the inner dental epithelial cells (with slow division rate) of the primary enamel knot occurred (Nadiri et al., 2004). Moreover, during this stage, a significant increase in the surface covered by the inner dental epithelium was also observed. This is partly due to cell proliferation which is stimulated by FGF4 and subsequently results to increasing the molar size (Nadiri et al., 2004). Finally, at the late bell stage, cytodifferentiation must have occurred, odontoblasts and ameloblasts progressively differentiated from

TABLE 2. Immunolocalization comparison of the signaling molecule FGF4 during the development of the first lower molar in three examined muroid rodents: house mouse (*Mus musculus*), golden hamster (*Mesocricetus auratus*) and Goodwin's brush-tailed mouse (*Calomyscus elburzensis*)

	Immunolocalization of FGF4		
Embryonic (E)/ postnatal (PN) days	*Mus musculus*	*Mesocricetus auratus*	*Calomyscus elburzensis*
E10	Some cells throughout the tissue	oral epithelium (I); underlying mesenchyme (W)	–
E12	Oral epithelium and the forming alveolar bone (I); underlying mesenchyme (W)	–	–
E13	Basement membrane (I); epithelium and mesenchyme (W)	dental papilla, inner and outer dental epithelia (I); dental mesenchyme (W)	Intense immunostaining throughout the tissue
E14	Oral epithelium (I); mesenchyme, inner and outer dental epithelia (W)	–	Oral epithelium and underlying mesenchyme (I)
E15	Inner and outer dental epithelia (I); mesenchyme (W)	–	–
E17	Dental papilla, inner and outer dental epithelia (I); dental mesenchyme (W)	–	Oral epithelium and basement membrane (I)
E22	–	–	Inner and outer dental epithelia (I); dental mesenchyme (W)
E25	–	–	Forming ameloblasts (I); stellate reticulum (W)
PN0	Odontoblasts and ameloblasts (I); stellate reticulum (W)	Pulp cavity at the tip of cusps (I); all other dental tissues (W)	Ameloblasts and odontoblasts (I); stellate reticulum and pulp cavity (W)

(I) and (W) refer to "Intense" and "Weak" immunostaining for FGF4, respectively.

the cusp tips toward the basal region of each cusp. Although three examined muroid species showed considerable differences in the timing of the bud to cap stage and also cap to bell stage transitions (Table 1), a similar pattern for FGF4 expression was observed in all three species (Table 2). This localization is in accordance with the considered role of FGF4 in tooth development and the cusp formation (see Jernvall and Thesleff, 2000) and likely contributes to the similarity in cusp patterns in Goodwin's brush-tailed mouse and the golden hamster molars and to some degree with that of the house mouse.

Teeth display considerable morphological variability and mammals show a wide range of modifications in their dentition which has allowed them to utilize a wide range of food sources. Among mammals, rodents have been studied as common experimental models for different aspects of dental development, especially for investigating the epithelial–mesenchymal interactions and patterns of tooth shape formation (Jernvall et al., 2000). Nevertheless, there are relatively few studies on tooth development among rodents other than laboratory mice, including a limited number of studies on rats (Masuhara et al., 1995; Santiago et al., 2013), voles (Keränen et al., 1998, 1999) and the silvery mole rat (Rodriguesa et al., 2011). Lesot et al. (1996) pointed out however, that the morphological variations and molecular changes during tooth development occur very fast and hence, tracing and providing the spatiotemporal order of events in the tooth serial sections is not easy.

The general evolutionary trends in dentition of mammals include the reduction in the number of teeth and an increasing complexity in cusp patterns (Harjunmaa, 2012). Brush-tailed mice of the family Calomyscidae are hypothesized to be the basal radiation of the mouse-like muroid rodents (Michaux et al., 2001; Steppan et al., 2004). Traditionally however, brush-tailed mice (*Calomyscus* spp.) were known as mouse-tailed hamsters due to similarity in molar patterns with hamsters (Cricetidae; Cricetinae) with which they were aligned (Thomas, 1905; Ellerman, 1941; Corbet, 1978). For instance, the only difference in the first lower molar shape between brush-tailed mice and golden hamsters is the presence of an additional cusp, the anterolabial conulid (Fig. 1). Wessels (1996) hypothesized that the similarity in dental morphology of brush-tailed mice and megacricetodontines, the presumed ancestor of Cricetidae including hamsters (Cricetinae), may be the result of convergent or parallel evolution. Slight changes in the genetic mechanisms defining the tooth shape could result in the appearance of new cusps. For example, slight temporal variations in the expression of regulatory genes could lead to changes in the formation time of secondary enamel knots and finally the evolution of additional cusps (Harjunmaa, 2012). Examination of development

and temporal pattern of the first lower molar formation in Goodwin's brush-tailed mouse and golden hamster, with an additional cusp of the first lower molar, revealed a slower development of molar pattern in the phylogenetically more primitive taxon (Calomyscidae) as compared to Cricetinae with slightly more complex molar pattern. The dentine and enamel matrix were completely formed in newborn pups (PN0) of golden hamster whereas the first lower molar was at an intermediate bell stage with only a defined layer of predentin in newborn pups of brush-tailed mice. Probably by the end of the first few days following birth in Goodwin's brush-tailed mouse, normal thickness of enamel matrix and dentin will be obtained.

Genes like *Fgf4* are thought to be more involved in regulating the patterning of species-specific cusps and tooth shape than other genes, such as *Msx1*, which regulates normal tooth development (Jernvall and Thesleff, 2000). Keränen et al. (1998) found that the molars of the house mouse (*Mus musculus*) and the east European vole (*Microtus rossiaemeridionalis*), with very different cusp patterns go through almost the same temporal pattern of developmental stages. Comparing the expression patterns of several developmental regulatory genes (such as *Bmp2*, *Bmp4*, *Fgf4*, and *Shh*) in these two species showed that similar molecular cascades have roles in the initiation of each cusp development and tooth crown formation. Although the transition to cap stage was faster in the vole, subsequently tooth morphogenesis diverged in these two species and the species-specific crown shapes was apparent at the bell stage (around E16). In the development of the vole molars, the molar length continued to increase faster than in house mouse molars, whereas the growth in height slowed down during cusp pattern formation. Keränen et al. (1998) also stated that *Fgf4* showed the closest correlation with the formation of secondary enamel knots and cusp patterning both in the house mouse and east European vole as compared with other genes examined.

A similar pattern for FGF4 expression was observed in golden hamster and Goodwin's brush-tailed mouse. Despite the differences in the timing of stages of the first lower molar morphogenesis between these two taxa, FGF4 was detected at the bud to cap stage, the cap to bell stage and then decreased during the late bell stage and cytodifferentiation. Weak immunostaining for FGF4 in the mesenchyme except for the tips of cusps during the bell stage, is in accord with the role of this signaling molecule in cusp formation. The subsequent decrease of FGF4 is correlated with the withdrawal of ameloblasts and odontoblasts from the cell cycle (Jernvall and Thesleff, 2000; Nadiri et al., 2004). The strong correlation in the expression of FGF4 with specific stages of lower molar morphogenesis further supports its regulatory function.

The generally weak immunostaining for FGF4 in the first lower molar of golden hamsters combined with the rapid transition through the bell stages (E12.5–13) likely masked the detection of the developmental events leading to an additional cusp in this taxon. Additional investigation on the role of FGF4 and other regulatory gene products could be useful for making more precise inferences about the embryonic development of cusp patterns between brush-tailed mice and hamsters. Future studies should consider examining other species of hamsters and plan on collecting a finer series of temporal samples between the caps to bell transition (E11.5-E13 in golden hamster).

CONCLUSION

The present study is the first analysis of the first lower molar development and FGF4 expression pattern in Goodwin's brush-tailed mice and golden hamsters in comparison with another muroid rodent, the house mouse, which has been a model organism for the study of molar development. Although embryonic development was approximately twice as long in the brush-tailed mouse as in the hamster, FGF4 expression pattern among the three species occurred at the same stages of tooth morphogenesis. Additional experiments are required for further investigation at other embryonic and postnatal days, and to analyze the expression and signaling effects of this and other regulatory molecule, for which little information are available. Moreover, further insights into the developmental mechanisms are needed to link the temporal differences in gene expression and morphogenetic events which are the causes of the differences in tooth shapes and diversification of molar tooth morphologies in muroid rodents.

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LITERATURE CITED

Corbet GB. 1978. The mammals of the palaeartic region: A taxonomic review. London: British Museum of Natural History.

Ellerman JR. 1941. The families and genera of living rodents. Vol. 2. London: British Museum of Natural History.

Hamidi K, Darvish J, Matin MM, Malikov VG. 2015. A field behavioral study of *Calomyscus elburzensis* (Rodentia: Calomyscidae): The effects of sexual and seasonal variations in the trapping success. Acta Zool Bulg 67:521–528.

Harjunmaa E. 2012. Tinkering with cusp patterning: developmental genetic mechanisms in mouse molar development. Published Ph.D. thesis in developmental biology program. Helsinki: Unigrafia Press. 63 pp.

Hillson S. 2005. Teeth: Cambridge manuals in archaeology. 2nd ed. New York: United States of America by Cambridge University Press. 388 pp.

Jernvall J, Keränen SVE, Thesleff I. 2000. Evolutionary modification of development in mammalian teeth: Quantifying gene expression patterns and topography. Proc Natl Acad Sci USA 97: 14444–14448.

Jernvall J, Kettunen P, Karavanova I, Martin LB, Thesleff I. 1994. Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: Non-dividing cells express growth stimulating *Fgf4* gene. Int J Dev Biol 38:463–469.

Jernvall J, Thesleff I. 2000. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 92:19–29.

Keränen SVE, Åberg T, Kettunen P, Thesleff I, Jernvall J. 1998. Association of developmental regulatory genes with the development of different molar tooth shapes in two species of rodents. Dev Genes Evol 208:477–486.

Keränen SVE, Kettunen P, Åberg T, Thesleff I, Jernvall J. 1999. Gene expression patterns associated with suppression of odontogenesis in mouse and vole diastema regions. Dev Genes Evol 209: 495–506.

Kettunen P, Thesleff I. 1998. Expression and function of FGFs-4, −8, and −9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. Dev Dyn 211: 256–268.

Lesot H, Vonesch JL, Peterka M, Tureckova J, Peterkova R, Ruch JV. 1996. Mouse molar morphogenesis revisited by three dimensional reconstructions. II. Spatial distribution of mitoses and apoptosis in cap to bell staged first and second upper molar teeth. Int J Dev Biol 40:1017–1031.

Luo ZX, Cifelli RL, Kielan-Jaworowska Z. 2001. Dual origin of tribosphenic mammals. Nature 409:53–57.

Maas R, Bei M. 1997. The genetic control of early tooth development. Crit Rev Oral Biol Med 8:4–39.

Masuhara K, Nakase T, Suzuki S, Takaoka K, Matsui M, Anderson HC. 1995. Use of monoclonal antibody to detect bone morphogenetic protein-4 (BMP-4). Bone 16:91–96.

Michaux J, Reyes A, Catzeflis F. 2001. Evolutionary history of the most species mammals: Phylogeny of muroid rodents. Mol Biol Evol 18:2017–2031.

Nadiri A, Kuchler-Bopp S, Haikel Y, Lesot H. 2004. Immunolocalization of BMP-2/-4, FGF4, and WNT10b in the developing mouse first lower molar. J Histochem Cytochem 52:103–112.

Nanci A. 2008. Ten Cate's oral histology: Development, structure, and function. St Louis, Missouri: Mosby Elsevier. 432 pp.

Rodriguesa HG, Marangonia P, Sumberab R, Tafforeauc P, Wendelend W, Viriota L. 2011. Continuous dental replacement in a hyper-chisel tooth digging rodents. Proc Natl Acad Sci USA 108: 17355–17359.

Santiago IMA, Regueira LS, Correia PG, Alcântara BRJ, Neto JE, Baratella-Evêncio L. 2013. Morphological aspects of dentin-pulp complex development in the offspring of rats treated with fluoxetine during pregnancy. Braz J Oral Sci 12:30–36.

Soderwall AL, Kent HA Jr, Turbyfill CL. Britenbaker AL 1960. Variation in gestation length and litter size of the golden hamster *Mesocricetus auratus*. J Gerontol 15:246–248.

Steppan SJ, Adkins RM, Anderson J. 2004. Phylogeny and divergence date estimates of rapid radiations in muroid rodents based on multiple nuclear genes. Syst Biol 53:533–553.

Thesleff I, Jalkanen M, Vainio S, Bernfield M. 1988. Cell surface proteoglycan expression correlates with epithelial-mesenchymal interactions during tooth morphogenesis. Dev Biol 129:565–572.

Thesleff I, Sharpe P. 1997. Signaling networks regulating dental development. Mech Dev 67:111–123.

Thesleff I, Vaahtokari A, Partanen AM. 1995. Regulation of organogenesis: Common molecular mechanisms regulating the development of teeth and other organs. Int J Dev Biol 39:35–50.

Thomas O. 1905. On a collection of mammals from Persia and Armenia, presented to the British Museum by Col. A. C. Bailward. Proc Zool Soc Lond 2:519–527.

Tucker A, Sharpe P. 2004. The cutting-edge of mammalian development: How the embryo makes teeth. Nat Rev Genet 5:499–508.

Vaahtokari A, Aberg T, Thesleff I. 1996. Apoptosis in the developing tooth: association with an embryonic signaling center and suppression by EGF and FGF-4. Development 122:121–129.

Volf VJ, Volf PP. 2003. Der mausartige zwerghamster (*Calomyscus bailwardi* Thomas 1905) und seine zucht menschenobhut. Zoologische Garten 73:147–157.

Wessels W. 1996. Myocricetodontinae from the Miocene of Pakistan. Proc Koninklijke Nederlandse Akademie van Wetenschappen 99(3–4):253–312.

Wood AE, Wilson RW. 1936. A suggested nomenclature for the cusps of the cheek teeth of rodents. J Paleo 10:388–391.

Woodburne MO, Rich TH, Springer MS. 2003. The evolution of tribospheny and the antiquity of mammalian clades. Mol Phylogenet Evol 28:360–385.