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Evolution and Development of the Mammalian Dentition: Insights From the Marsupial *Monodelphis domestica*

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To understand developmental mechanisms of evolutionary change, we must first know how different morphologies form. The vast majority of our knowledge on the developmental genetics of tooth formation derives from studies in mice, which have relatively derived mammalian dentitions. The marsupial *Monodelphis domestica* has a more plesiomorphic heterodont dentition with incisors, canines, premolars, and molars on both the upper and the lower jaws, and a deciduous premolar. The complexity of the *M. domestica* dentition ranges from simple, unicusped incisors to conical, sharp canines to multicusped molars. We examine the development of the teeth in *M. domestica*, with a specific focus on the enamel knot, a signaling center in the embryonic tooth that controls shape. We show that the tooth germs of *M. domestica* express fibroblast growth factor (FGF) genes and Sprouty genes in a manner similar to wild-type mouse molar germs, but with a few key differences. *Developmental Dynamics 240:232–239, 2011*. © **2010 Wiley-Liss, Inc.**

Key words: tooth development; enamel knot; FGF; Sprouty; heterodont; marsupial; Shh; tribosphenic

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INTRODUCTION

A central question in evolutionary morphology and developmental biology is how diversity in the shape and size of structures is achieved. The mammalian dentition has proved to be a productive system to study the developmental generation of complex structures and to model mechanisms of morphological change. Thus far, most molecular and genetic studies of dental development have focused on rodents, in particular the mouse *Mus musculus*. *Mus*, however, has a fairly uniform dentition, with little variation in tooth type along the dental arcade (only incisors and molars). Most mammals possess a more heterodont dentition, in which there are several distinct types of teeth. Tooth shapes in mammals can range from a simple conical shape (such as canines) to the complex arrangements of cusps seen in molars. To understand the generation of diversity of tooth types across mammals broadly it is critical to study an animal that possesses a more typical mammalian heterodont dentition.

In this study, we present data on the expression of major genes known to be important in tooth development and patterning in the gray short-tailed opossum *Monodelphis domestica* (Fig. 1a). *M. domestica* is a small, easily bred animal that has been used extensively as a research organism (Keyte and Smith, 2008) and whose genome has been sequenced recently (reviewed in Mikkelsen et al., 2007). Importantly, *M. domestica* has a complete heterodont dentition, including incisors, canines, premolars and molars, as well as a deciduous premolar (although all premolars are generally considered to be deciduous (Luckett, 1993), only the third is replaced in marsupials). *M. domestica* also

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retains a tribosphenic dentition, considered a key innovation of some of the earliest mammals (Luo, 2007), in which the cusps of the upper teeth occlude with a talonid basin formed by smaller cusps on the lower teeth (Fig. 1a). Data from *M. domestica* therefore can provide critical information on patterning heterodont dentition, deciduous dentition, and the primitive tribosphenic condition.

GENETICS OF MOUSE DENTAL DEVELOPMENT

Early tooth development establishes the type of tooth at a specific location and the morphology or shape of the tooth (Jernvall, 1995). Tooth development proceeds through a series of morphogenetic movements and signaling interactions between ectodermal epithelium and neural crest-derived ectomesenchyme. The first indication of tooth development is the formation of the dental lamina, a thickening of the epithelium that marks the future dental arch. A dental placode forms as an epithelial thickening along the dental lamina at the future position of the tooth. This thickening grows and begins to invaginate into the underlying ectomesenchyme, which then condenses around the epithelium to form a tooth bud. The epithelium then folds and extends farther into the mesenchyme, forming a cap and then a bell stage tooth germ. The tooth itself is composed of both layers with the ameloblasts formed from the epithelium and the odontoblasts differentiating from the ectomesenchyme. The ameloblasts deposit enamel and the odontoblasts secrete dentine (reviewed in Jernvall and Thesleff, 2000). An embryonic signaling center that controls tooth shape, the enamel knot (EK), is induced at the cap stage (Jernvall et al., 1994; Fig. 1b). The EK is thought to control tooth shape by controlling the differential growth and folding of the epithelium (Jernvall and Thesleff, 2000).

The primary EK is a transient, nonproliferative cluster of epithelial cells that expresses several signaling molecules and has been shown to be essential for mammalian tooth development (Jernvall et al., 1994; Vaahtokari et al., 1996). *Sonic hedgehog* (*Shh*) expression in the EK of mammalian tooth germs has been documented in placental mammals, including mouse (Vaahtokari et al., 1996), vole (Keränen et al., 1998), shrew (Yamanaka et al., 2007), and ferret (Järvinen et al., 2009). Evidence from wild-type and mutant mice suggests that a functional EK is induced/maintained by signaling between epithelium and mesenchyme by means of fibroblast growth factors (FGFs; Kettunen et al., 2000) and that this signaling is modulated by negative feedback regulators of FGF and other receptor tyrosine kinase (RTK) signaling, encoded by Sprouty genes (Klein et al., 2006).

Members of the FGF family of secreted intercellular signaling molecules affect organ development through regulating cell proliferation and differentiation (reviewed in Martin, 1998). FGFs have important roles in the development of several vertebrate organs, including the lungs (Peters et al., 1994), the kidneys (Karavanova et al., 1996), the limbs (reviewed in Wilkie et al., 2002), the brain (reviewed in Iwata and Hevner, 2009), the hair (Hebert et al., 1994), the turtle shell (Cebra-Thomas et al., 2005; Moustakas, 2008), the feathers (Widelitz et al., 1996), and the teeth (reviewed in Jernvall and Thesleff, 2000). FGFs exert their biological effects through four high-affinity tyrosine kinase receptors (FGFR1–4; reviewed in Wilkie et al., 2002). Studies on mice mutant for FGF receptors have shown that FGFs are necessary for tooth morphogenesis to proceed from the bud stage to the cap stage (Celli et al., 1998; De Moerlooze et al., 2000).

Feedback control of biological signaling pathways is an important mechanism for ensuring the spatial and temporal regulation of cell proliferation and differentiation. Sprouty proteins are negative-feedback regulators that inhibit signaling by RTKs (reviewed in Kim and Bar-Sagi, 2004). The *spry* gene was first identified as a negative feedback regulator of FGF-mediated tracheal branching in *Drosophila* (Hacohen et al., 1998) and subsequent studies have shown that *spry* regulates other RTK signaling pathways as well (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). The mammalian genome contains four *Sprouty* genes (de Maximy et al., 1999), with *Spry* genes *1*, *2*, and *4* having expression in several organs in the developing mouse embryo (Minowada et al., 1999; Zhang et al., 2001). The expression patterns of these *Sprouty* genes suggest roles in epithelial–mesenchymal signaling interactions (Zhang et al., 2001).

Comparing Mouse Dental Developmental Genetics to *Monodelphis*

Most investigations of the developmental genetics of tooth morphogenesis have used mice as a model system. Mice are placental mammals with a reduced dentition that is composed of incisors and molars that are separated by a toothless diastema. Data from studies on rodents with different molar tooth morphologies (mice; [Jernvall et al., 1994; Vaahtokari et al., 1996; Kettunen and Thesleff, 1998; Kettunen et al., 2000] and voles [Keränen et al., 1998]) have shown that the EKs of the molar tooth primordia express some of the same developmental regulatory genes. However, we know very little regarding the developmental expression of other tooth classes.

In this study, we examine expression patterns of genes shown to be important in generating mouse molar teeth in mice in all tooth types in *M. domestica*. We first examine the timing of dental lamina formation in *M. domestica*. *Shh* and *Fgf8* are expressed in the dental lamina in mouse (Bitgood and McMahon, 1995; Kettunen and Thesleff, 1998). *Shh* is also expressed in the dental lamina of snakes (Buchtova et al., 2008), shrews (Yamanaka et al., 2007), and catsharks (Smith et al., 2009), suggesting a strong conservation at this early stage of tooth development and a role in the generation of replacement teeth.

We then focus on genes that regulate morphogenesis at the cap stage of tooth development, when shape is being controlled. Specifically, we describe the expression patterns of *Fgf-3*, *–10*, and *–4*, *Shh*, and *Spry-2* and *-4* in cap-stage *Monodelphis* tooth germs. FGF signaling is thought to induce/maintain a functional EK (Klein et al., 2006) and control cell

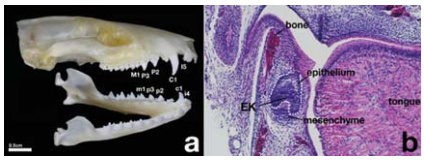

Fig. 1. **a:** Skull of *Monodelphis domestica* (UCMP 197900) showing the adult morphology and relative position of the tooth germs investigated, with the exception of the third premolars, which are the replacement rather than the deciduous teeth. M1, first upper molar; P3, third upper premolar; P2, second upper premolar; C1, upper canine; I5, fifth upper incisor; m1, first lower molar; p3, third lower premolar; p2, second lower premolar; c1, lower canine; i4, fourth lower incisor. **b:** Histological section of a *M. domestica* cap stage lower molar stained with hematoxylin and eosin, illustrating the morphology of the epithelium and mesenchyme. EK, enamel knot.

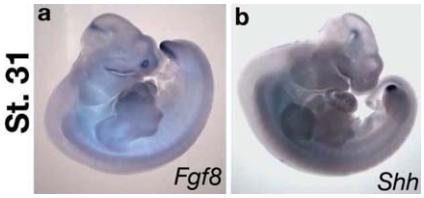

Fig. 2. Formation of the dental lamina in *Monodelphis domestica*. **a,b:** Whole-mount embryos assayed for *Fgf8* (a) and *Shh* (b) expression by in situ hybridization, showing the expression of these genes in the dental lamina at stage 31.

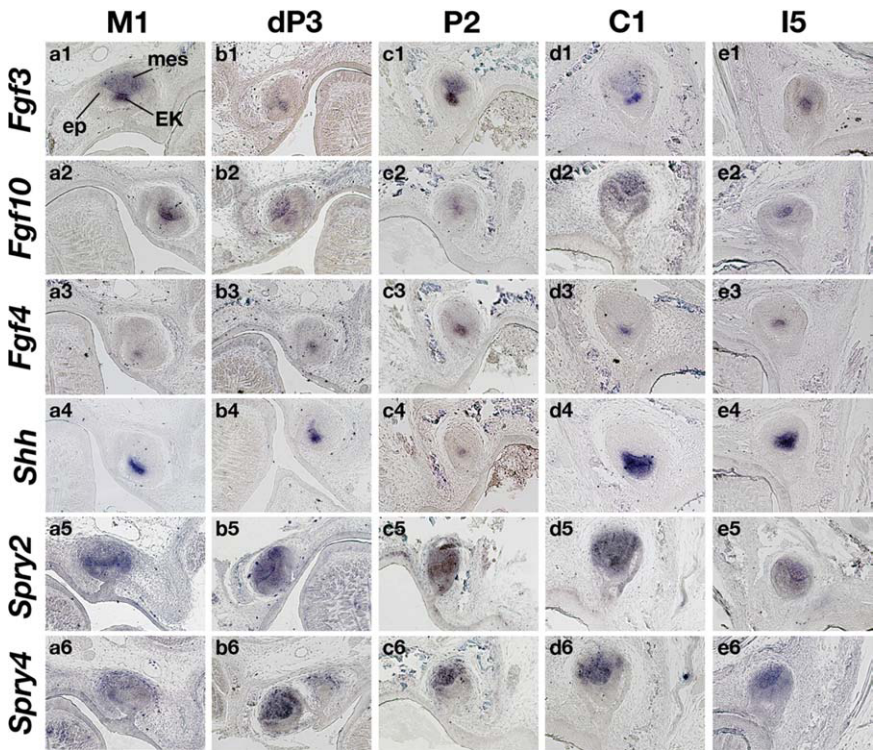

Fig. 3. **a–e:** Gene expression in cap-stage tooth germs of the upper jaw (maxillary arch) of *Monodelphis domestica* postnatal specimens. Tooth class/germ is indicated at top and gene examined is on left. *Fgf3* is expressed in the enamel knot (EK) and mesenchyme of all tooth germs (a1–e1). *Fgf10* is expressed in the EK and mesenchyme of M1 (a2), dP3 (b2), and P2 (c2), in the epithelium and mesenchyme of C1 (d2), and the mesenchyme of I5 (e2). *Fgf4* is expressed in the EK (a3–e3). *Shh* is expressed in the EK (a4–e4). *Spry2* is expressed in the epithelium and mesenchyme (a5–e5). *Spry4* is expressed in the epithelium and mesenchyme (a6–e6). ep, epithelium; mes, mesenchyme.

proliferation around the EK (Jernvall and Thesleff, 2000). *Sprouty* genes are expressed in developing mouse molar and incisor teeth, as well as in the toothless diastema (Klein et al., 2006, 2008). In mouse molar tooth germs, *Spry2* is predominant in the epithelium and *Spry4* is predominant in the mesenchyme at the cap-stage of tooth development (Klein et al., 2006). Null mice mutant for *Spry* genes *2* or *4* develop a tooth in the diastema region (Klein et al., 2006). The loss of Sprouty gene expression in the diastema buds results in an increase in FGF and RTK signaling in the diastema bud epithelium and the formation of a functional EK and tooth (Klein et al., 2006). The development of this mutant tooth is intriguing because the morphology of this diastema tooth is similar to that of a premolar and could therefore reflect the normal development of premolars. We describe expression in incisor, canine, deciduous and permanent premolar, and molar teeth.

As FGFs are necessary for the completion of tooth morphogenesis (Celli et al., 1998; De Moerlooze et al., 2000), we expect that basic conditions of FGF signaling and its modulation are conserved among the different classes of teeth. However, we hypothesize that we will find differences in the expression patterns of these genes in the different tooth classes that are attributable to different tooth morphologies. Our study is thus the first to examine these genes, known to be central in patterning mammalian tooth form, in the full range of mammalian tooth classes, as well as in both deciduous and permanent adult teeth.

RESULTS

Formation of the Dental Lamina in *M. domestica*

The first placodes, or thickenings of the dental lamina, have been observed for the deciduous third premolars in neonates (van Nievelt and Smith, 2005), all other teeth develop later. The odontogenic epithelium, however, is induced in the embryo (Fig. 2) and both *Fgf8* and *Shh* are expressed in the dental lamina of embryonic stage 31 (approximately 2 days before birth; Fig. 2a,b).

Expression of Fgf, Shh, and Sprouty Genes in the Different *M. domestica* Tooth Classes

We examined the cap stage of tooth development in postnatal specimens. The cap stage occurs at 3 days postnatal (3P) for tooth germs dP3 and dp3, 5P for M1 and m1, 7P–8P for C1 and c1, 8P for P2 and p2, 9P–11P for i4, and 11P–13P for I5 (van Nievelt and Smith, 2005; here). We describe gene expression patterns of *Fgf3*, *Fgf10*, *Fgf4*, *Shh*, *Spry2*, and *Spry4* in cap stage germs of different tooth classes.

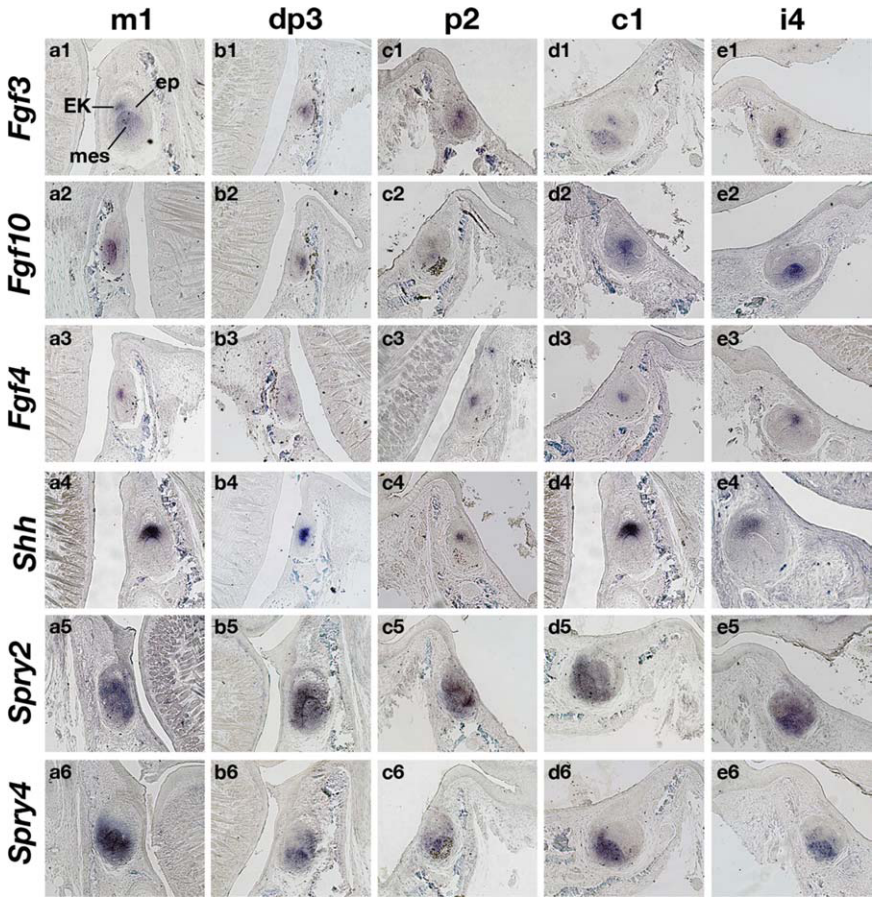

Fig. 4. **a–e:** Gene expression in cap-stage tooth germs of the lower jaw (mandibular arch) of *Monodelphis domestica* postnatal specimens. Tooth class/germ is indicated at top and gene examined is on left. *Fgf3* is expressed in the enamel knot (EK) and mesenchyme of all tooth germs (a1–e1). *Fgf10* is expressed in the EK and mesenchyme of m1 (a2), dp3 (b2), and p2 (c2), in the epithelium (and EK) and mesenchyme of c1 (d2), and the mesenchyme of i4 (e2). *Fgf4* is expressed in the EK (a3–e3). *Shh* is expressed in the EK (a4–e4). *Spry2* is expressed in the epithelium and mesenchyme (a5–e5). *Spry4* is expressed in the epithelium and mesenchyme (a6–e6).

Upper and Lower First Molars

Fgf3 and *Fgf10* are expressed in the mesenchyme and EK of the first upper molar tooth germ (M1), whereas *Fgf4* is exclusively in the EK (Fig. $3a_{1\text{-}3}$). *Shh* is expressed in the EK in a broader pattern than *Fgf4* (Fig. $3a_4$). *Spry2* is expressed in the epithelium and the mesenchyme (Fig. $3a_5$). *Spry4* is mostly expressed in the mesenchyme in M1, but also lingually in the epithelium (Fig. $3a_6$).

As in the upper germ, *Fgf3* and *Fgf10* are expressed in the mesenchyme and EK of the first lower molar tooth germ (m1; Fig. $4a_{1\text{-}2}$). *Fgf4* and *Shh* are expressed in the EK with the expression of *Shh* being broader than that of *Fgf4* (Fig. $4a_{3\text{-}4}$). *Spry2* and *Spry4* are expressed in both the epithelium and mesenchyme of m1 (Fig. $4a_{5\text{-}6}$).

Upper and Lower Premolars

Fgf3 and *Fgf10* are expressed in the mesenchyme and EK of the upper deciduous third premolar (dP3; Fig. $3b_{1\text{-}2}$). *Fgf4* and *Shh* are expressed in the EK, with *Shh* again having a broader domain of expression than *Fgf4* (Fig. $3b_{3\text{-}4}$). *Spry2* and *Spry4* are both expressed in the epithelium and mesenchyme of dP3; *Spry2* is expressed throughout the epithelium, whereas *Spry4* expression is stronger in the tips of the epithelium (Fig. $3b_{5\text{-}6}$).

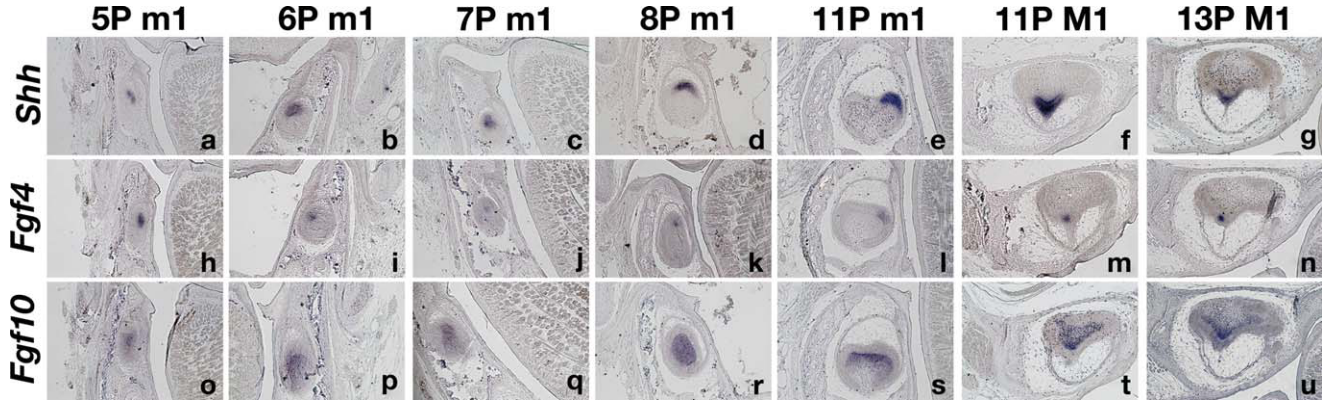

Fig. 5. The development of the primary enamel knot (PEK) and secondary enamel knots (SEKs) in the tribosphenic molars of *Monodelphis domestica*. **a–d:** Tooth and days postnatal are indicated at top and gene examined is on left. **e–g:** *Shh* is expressed in the PEK of the lower first molar (m1) from 5P–8P (a–d). *Shh* is expressed in the SEKs of m1 (e) and M1 (f,g). **h–k:** *Fgf4* is expressed in the PEK of the lower first molar (m1) from 5P–8P. **l–n:** *Fgf4* is expressed in the SEKs of m1 (l) and M1 (m,n). **o–q:** *Fgf10* is expressed in the PEK and the mesenchyme of the lower first molar (m1) from 5P–7P. **r–u:** *Fgf10* is expressed only in the mesenchyme of m1 at 8P (r) and M1 at 11P (s) and 13P (t,u).

TABLE 1. Summary of Gene Expression Patterns in Mouse and Opossum Cap-Stage Tooth Germs

	M. musculus molars		*M. domestica* incisors		*M. domestica* canines		*M. domestica* premolars		*M. domestica* Molars	
Gene	Epithelium	Mesenchyme	Epi	Mes	Epi	Mes	Epi	Mes	Epi	Mes
Shh	x		x		x		x		x	
Fgf4	x		x		x		x		x	
Fgf3	x	x	x	x	x	x	x	x	x	x
Fgf10		x		x	x	x	x	x	x	x
Spry2	x		x	x	x	x	x	x	x	x
Spry4		x	x	x	x	x	x	x	x	X

In the lower deciduous third premolar (dp3), *Fgf3* and *Fgf10* are expressed in both the mesenchyme and EK (Fig. 4b$_{1-2}$), whereas *Fgf4* and *Shh* expression is confined to the EK (Fig. 4b$_{3-4}$). As in dP3, *Spry2* and *Spry4* expression is seen in both the epithelium and mesenchyme of dp3, with *Spry2* expression seen throughout the epithelium and *Spry4* epithelial expression being stronger in the tips of the epithelium (Fig. 4b$_{5-6}$).

We also examined the second premolars that form part of the permanent dentition. In both the upper (P2) and lower (p2) second premolars, *Fgf3* and *Fgf10* are expressed in the mesenchyme and the EK (Fig. 3c$_{1-2}$, 4c$_{1-2}$). *Fgf4* and *Shh* are expressed in the EK of P2 (Fig. 3c$_{3-4}$) and p2 (Fig. 4c$_{3-4}$). *Spry2* expression is in the epithelium and mesenchyme of P2 (Fig. 3c$_5$) and p2 (Fig. 4c$_5$). *Spry4* is expressed in the mesenchyme and weakly in the epithelium of P2, with a lingual bias as in M1 (Fig. 3c$_6$). *Spry4* is expressed in the mesenchyme and the epithelium of p2, with the mesenchymal expression being relatively stronger (Fig. 4c$_6$).

Upper and Lower Canines

Fgf3 is expressed in the EK and mesenchyme of both the upper canine (C1; Fig. 3d$_1$) and lower canine (c1; Fig. 4d$_1$). *Fgf10* is expressed in both the mesenchyme and epithelium of C1 and c1, with the mesenchymal expression being stronger than the epithelial expression (Figs. 3d$_2$, 4d$_2$). The expression of *Fgf10* is seen diffusely in C1 (Fig. 3d$_2$) and has strong expression in the EK of c1 (Fig. 4d$_2$). *Fgf4* and *Shh* are expressed in the EK of C1 and c1, with *Shh* expression being broader than *Fgf4* (Fig. 3d$_{3-4}$, 4d$_{3-4}$). *Spry2* is expressed in the mesenchyme and the epithelium surrounding the mesenchyme of C1 and c1 (Fig. 3d$_5$, 4d$_5$). *Spry4* is expressed in the mesenchyme of C1 and c1 and in the tips of the epithelium (Fig. 3d$_6$, 4d$_6$).

Upper Fifth and Lower Fourth Incisors

Fgf3 is expressed in the EK and mesenchyme of the upper fifth incisor (I5; Fig. 3e$_1$).

Fgf10 is expressed in the mesenchyme of I5 (Fig. 3e$_2$). *Fgf4* and *Shh* are expressed in the EK of I5 (Fig. 3e$_{3-4}$). *Spry2* is expressed in the epithelium and mesenchyme of I5 (Fig. 3e$_5$) and *Spry4* is expressed in the mesenchyme of I5 (Fig. 3e$_6$).

In the lower fourth incisor (i4), *Fgf3* is expressed in the mesenchyme and the EK (Fig. 4e$_1$), *Fgf10* is expressed in the mesenchyme (Fig. 4e$_2$), and *Fgf4* and *Shh* are expressed in the EK (Fig. 4e$_{3-4}$). *Spry2* is expressed in the epithelium and mesenchyme of i4 (Fig. 4e$_5$) and *Spry4* is expressed strongly in the mesenchyme and weakly in the epithelium of i4 (Fig. 4e$_6$).

Primary and Secondary Enamel Knots in the Tribosphenic Molars

The primary enamel knot at the tip of the protoconid in the *M. domestica* lower molar persists longer than the primary enamel knot of the lower-cusped talonid (Jernvall, 1995). We, therefore, examined the development of the lower molar primary enamel knot (PEK) through the induction of the secondary enamel knots (SEK). Secondary enamel knots form later in tooth development at the cusp tips (Jernvall et al., 1994).

The PEK at the tip of the protoconid in *M. domestica* persists until 8P (Jernvall, 1995) and expresses *Shh* and *Fgf4* (Fig. 5a–d,h–k). SEKs of the lower molar also express *Shh* and *Fgf4* (Fig. 5e,l). The epithelial expression of *Fgf10* in the PEK persists through 7P (Fig. 5o–q). At later stages, *Fgf10* is no longer expressed in the dental epithelium; however, the mesenchymal expression of *Fgf10* is maintained (Fig. 5o–s).

Shh and *Fgf4* are also expressed in the SEKs of the upper molar (Fig. 5f,g,m,n). *Fgf10* is expressed in the mesenchyme, but not in the epithelium, of the developing upper molars during SEK formation (Fig. 5t,u).

DISCUSSION

Expression of *Shh*, *Fgf*, and *Sprouty* Genes in Tooth Development Is Conserved Between Marsupial and Placental Mammals, as Well as Across Tooth Classes

We have shown that key genes that regulate tooth morphogenesis in mouse are also expressed in the developing tooth germs of the marsupial *Monodelphis domestica*. Many of the domains of expression of the *Fgf*, *Shh*, and *Sprouty* genes that we examined in *M. domestica* tooth germs are conserved with the expression patterns seen in mouse (Table 1). *Fgf3*, *Fgf4*, and *Shh* are expressed in the enamel knots of *M. domestica* and mouse, and *Fgf3* and *Fgf10* are expressed in the mesenchymal papillae (Jernvall et al., 1994; Kettunen and Thesleff, 1998; Kettunen et al., 2000). In mouse incisor and molar tooth germs, *Spry2* is expressed predominantly in the epithelium and *Spry4* is expressed

predominantly in the mesenchyme at the cap-stage of tooth development (Klein et al., 2006, 2008). In contrast, *Spry* genes *2* and *4* are more broadly expressed in many of the tooth germs of *M. domestica* than mouse, having both epithelial and mesenchymal domains of expression. Because the epithelial–mesenchymal cross-talk between these *Fgfs* and *Sprouty* genes induces/maintains *Shh* expression in the EK at the cap stage of tooth development (Klein et al., 2006), we hypothesize that the function of FGFs in cap stage tooth germs is conserved between marsupials and placentals.

Fgf10 Has Epithelial Domains of Expression in *M. domestica*

In contrast to the many genes with conserved expression, we found differences in *Fgf10* expression patterns in *M. domestica* tooth germs. In mice, *Fgf10* is only expressed in the mesenchyme (Kettunen et al., 2000); for *M. domestica*, we observed *Fgf10* expression in the enamel knot and mesenchymal papilla of the canine, premolar, and molar teeth. This is the first observation of epithelial *Fgf10* expression in tooth development, and given its variation in degree of expression, we hypothesize that it underlies variation in tooth shape.

The enamel knot is an embryonic signaling center that controls tooth shape (Jernvall et al., 1994) and is essential for tooth morphogenesis. FGF10 has been shown to stimulate cell proliferation in cultured mouse molar dental epithelium (Kettunen et al., 2000) and to regulate cell survival of incisor epithelium (Ohuchi et al., 2000; Harada et al., 2002). Previous studies (Jernvall, 1995) have shown that the epithelium of *M. domestica* lower molar tooth germs grows faster relative to the mesenchyme. Experimental evidence, models of pattern formation, and simulation analyses have shown that differences in tooth morphology result from differences in epithelial growth (Keränen et al., 1998; Jernvall et al., 2000; Salazar-Ciudad and Jernvall, 2002, 2004). In particular, the sharpness of tooth cusps has been proposed to be affected by changes in the rate of epithelial growth (Salazar-Ciudad and Jernvall, 2010). Therefore, we hypothesize that the expression of *Fgf10* in the epithelium of canine tooth germs and in the primary enamel knots of premolar and molar tooth germs controls the faster rate of epithelial growth in these teeth in *M. domestica* and that this has led to differences in tooth morphology, such as cusp height and sharpness.

Sproutys 2 and *4* Are Expressed in Both the Epithelium and Mesenchyme of *M. domestica*

In mice incisor and molar cap-stage tooth germs, *Spry2* is expressed in the epithelium and *Spry4* is expressed in the mesenchyme (Klein et al., 2006, 2008). Because *Spry2*-null and *Spry4*-null mice develop a tooth in the diastema, immediately anterior to the lower first molar (Klein et al., 2006), it would be reasonable to hypothesize that these genes contribute to the suppression of premolar teeth and the formation of the diastema in mice, as *Spry2*-null and *Spry4*-null mice develop a tooth in the diastema, immediately anterior to the lower first molar (Klein et al., 2006). Our results are not consistent with this hypothesis, though, as we see *Spry2* and *Spry4* expression in both the epithelial and mesenchymal compartments of all tooth classes. Therefore, *Spry2* or *Spry4* expression does not necessarily lead to a diastema or suppression of premolar teeth. However, expression patterns for *Spry2* and *Spry4* in mice and *M. domestica* do differ. Unlike mice, in *M. domestica* both genes are expressed in both the mesenchyme and epithelium, and it is possible that these expression patterns may be important in maintaining a full heterodont dentition.

CONCLUSION

This study is the first study of gene expression patterns in the complete dentition of a mammal with a fully heterodont dentition, and provides critical information about the generation of dental diversity. First, we show that the role of many of the genes studied appears to be conserved, not only evolutionarily but across tooth classes of different dental types. Furthermore, we see little difference in patterning in the teeth considered deciduous or permanent. However, we describe one critical difference from previous studies, observed here for the first time. Teeth that have exceptionally sharp and tall cusps, specifically the canine, premolars, and molars, also have epithelial expression in the primary enamel knots of *Fgf10*. In mice, and in tooth germs that form relatively low cusps in *Monodelphis* (the incisors), *Fgf10* expression is limited to the mesenchyme. We propose that the epithelial expression of *Fgf10* leads to increased epithelial proliferation and therefore to increased height.

In addition, our data may contribute to discussions on the generation of the mammalian dental diastema. Whereas previous studies suggest that the expression of *Spry2* and *Spry4* is important in suppressing dental development in the diastema region, we observed these genes in all tooth germs. Our data thus suggest that Sprouty expression does not necessarily lead to diastema formation. Finally, our results emphasize the need for comparative data, and in particular data on the plesiomorphic condition, before we can fully understand the generation and evolution of tooth shape in mammals.

EXPERIMENTAL PROCEDURES

Specimens

M. domestica is a member of the family Didelphidae and is found throughout the northern two-thirds of South America (Streilein, 1982; Nowak, 1999; Macrini, 2004). The specimens used in this study were collected from a captive breeding colony maintained at Duke University by K.K.S. (Keyte and Smith, 2008). *M. domestica* young are born after 14.5 days of gestation, begin to detach from the teat 10–12 days after birth, and are weaned between 50 and 60 days after birth (Smith, 2006). The age of specimens is given in embryonic stages (Mate et al., 1994) or days postnatal with day of birth considered day 0P. The basic elements and timing of tooth development in *M. domestica* have been described in detail (van Nievelt and Smith, 2005). Postnatal specimens used in this study ranged from three

days postnatal to thirteen days postnatal to capture the cap stage of all dental classes (van Nievelt and Smith, 2005). All animal care was approved by the Duke University Institutional Animal Care and Use Committee in accordance with the established guidelines (National Research Council, 1996).

The upper dentition is denoted with a capital letter, the lower dentition with a lower-case letter. For example, the first upper molar is denoted "M1" and the second lower premolar is denoted "p2". Although all premolars in marsupials are generally considered to be of the deciduous generation (Luckett, 1993), the first and second are not replaced and contribute to the adult dentition. In marsupials, only the third premolar has a deciduous tooth that is later replaced by a second generation. Deciduous teeth are denoted by the prefix, "d".

M. domestica retains the primitive marsupial dental formula consisting of five upper and four lower incisors, one upper and one lower canine, three upper and three lower premolars, and four upper and four lower molars in each jaw quadrant (I 5/4, C 1/1, P 3/3, M 4/4; Fig. 1a). The incisors in *M. domestica* are simple, unicusped teeth that are flattened medio-laterally. The canines are the tallest teeth of the dental arcade and are formed by a single cusp. The lower premolars are composed of the paraconid, protoconid, and metaconid, where the protoconid is the tallest cusp. The upper premolars have three cusps, the paracone, protocone, and metacone, where the paracone is the largest cusp. The deciduous third premolar on both the lower and upper jaws is molariform. In the lower molars, the cusps of the talonid basin (hypoconid, entoconid, and hypoconulid) are relatively lower than the cusps of the trigonid (protoconid, paraconid, and metaconid). The upper molars are composed of three trigon cusps, the paracone (the largest cusp), protocone, and metacone, and four stylar cusps.

Isolation of Opossum Genes

Total RNA extraction was performed using the Aurum Total RNA kit (Bio-Rad, Hercules, CA). cDNA was generated using the Omniscript Reverse Transcriptase Kit (QIAGEN, Valencia, CA). Polymerase chain reaction (PCR) was used to isolate genes using platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). Fragments were cloned into the pCR4Blunt-TOPO vector (Invitrogen) using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). Orthologous genes were identified by BLAST and sequence alignment comparison in SeAl v2.0a11 (Rambaut, 1996). The aligned data matrices included orthologous and paralogous sequences of multigene families to identify homologues. The sequences for *Fgf8* (387 base pairs), *Fgf4* (381 bp), *Fgf3* (145 bp), *Spry2* (369 bp), and *Spry4* (535 bp) are deposited in GenBank under the following accession numbers: *Fgf8* GU984788, *Fgf4* GU984791, *Fgf3* GU984787, *Spry2* GU984789, and *Spry4* GU984790.

In Situ Hybridization

RNA probes were labeled with digoxigenin-11-UTP (Roche, Indianapolis, IN). *M. domestica Fgf10* and *Shh* plasmids were kindly provided by A. Keyte (GenBank accession numbers *Fgf10* GU593350 and *Shh* GU593352). Embryonic specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in methanol, and stored at −20°C. Whole-mount in situ hybridization was performed following Sive et al. (2000) with modifications found in Moustakas (2008). Postnatal specimens were fixed in a formol-alcohol fixative (Lillie, 1965; ethanol, formaldehyde, glacial acetic acid), dehydrated in ethanol, and stored at −20°C. Marsupial neonates have an extremely thick epidermis, which was removed before specimens were embedded in paraffin wax. Postnatal specimen heads were sectioned at 7–8 μm and the in situ hybridization on paraffin-embedded tissue sections was performed following Lescher et al. (1998) with modifications in Moustakas (2008). Hematoxylin and eosin staining was performed following Presnell and Schreibman (1997).

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