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Cell proliferation and tissue dynamics during development of teeth and tooth related palatal rugae

Proliferace a dynamika tkání během vývoje zubů a jim příbuzných patrových lišt

Type of final work: PhD dissertation

Supervisor: Renata Peterková, M.D., PhD.
Co-supervisor: Abigail Tucker, PhD.

Praha 2011

Declaration

I declare that I elaborated this present thesis by my own and I referred all relevant sources of used literature.
I also declare that neither this work nor its substantial part have been submitted to obtain another or the same academic degree.

Praha, 3. 8. 2011

Michaela Rothová

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Abstract

My PhD thesis has addressed specific questions regarding cell proliferation and tissue dynamics in three key areas of craniofacial development: during suppression and revitalization of tooth buds that develop in the mouse embryonic dentition as rudiments of lost premolars; during dental papilla and follicle formation of the first mouse molar; and during origin of palatal rugae on the mouse hard palate.

By evaluation of cell proliferation, we recorded a change in the proliferation pattern along the cheek region of the mandible between less and more advanced embryos at embryonic day 13.5. Thus during the time period, when the development of the large mouse rudimental premolar primordium (R2) is stopped, we showed that the arrest of the rudiment R2 is caused by exhibiting low rate of cell proliferation and high rate of apoptosis. When Sprouty gene signalling is disrupted, the premolar primordium shows rates of proliferation and apoptosis similar to the growing first molar. The R2 subsequently revitalizes and develops into a supernumerary tooth in front of the first molar. Furthermore, we discovered that the dental mesenchyme is very dynamic tissue during bud and cap stages of tooth development and that the dental papilla of the first molar originates only from a restricted region of the dental mesenchymal cells. We also proved that the influx of non-neural crest derived cells into the dental papilla is caused by immigration of mesoderm-derived endothelial cells, which starts at a specific time point, the embryonic day 15.0. Cell proliferation appeared as an important factor in palatal rugae origin and spacing. We observed high cell proliferation in regions in between palatal rugae and a burst of cell proliferation in the region of a new forming ruga.

This PhD work brings new important knowledge on the origin and patterning of epithelial appendages – experimentally proving the appearance and fate of the rudimental premolar primordia in the mouse dentition; proposing a new model of mouse dental papilla origin and indicating the mechanisms behind the origin and spacing of palatal rugae in mouse.

Key words: mouse, development, tooth, rudiment, dental mesenchyme, papilla, follicle, ruga.

Abstrakt

V této dizertační práci jsem se zaměřila na otázky týkající se buněčné proliferace a dynamiky tkání u tří klíčových oblastí obličejového vývoje: během potlačení nebo revitalizace zubních pupenů, které se vyvíjejí v myší embryonální dentici jako pozůstatky ztracených premolárů; během vzniku zubní papily a zubního folikulu prvního myšího moláru; a během vzniku patrových lišt na ústním povrchu tvrdého patra u myši.

Buněčnou proliferaci v tvářové oblasti dolní čelisti jsme porovnávali u méně a více vývojově pokročilých myších zárodků na embryonálním dni 13,5, v období předpokládané zástavy růstu premolárového rudimentu R2. Ukázali jsme, že růst rudimentu R2 je potlačen v důsledku nízké míry buněčné proliferace a vysoké míry apoptózy. Zajímavé bylo, že při narušení Sprouty signalizace, R2 rudiment vykazoval podobnou míru proliferace a apoptózy jako progresivně rostoucí první molár. V důsledku těchto změn se R2 rudiment u Sprouty 2 mutantních myší revitalizoval a vyvinul v nadpočetný zub před prvním molárem. Dále jsme objevili, že během stádia zubního pupenu a pohárku, je zubní mezenchym velmi dynamická tkáň, a že zubní papila prvního myšího moláru vzniká pouze z omezené populace buněk původní kondenzace zubního mezenchymu. Studovali jsme identitu buněk zubní papily, které nejsou odvozené od buněk neurální lišty, a prokázali jsme, že jsou jimi mesodermové endothelové buňky, které začínají imigrovat do zubní papily na embryonálním dni 15,0. Buněčná proliferace se ukázala jako významný faktor během vzniku a rozmístění patrových lišt. Prokázali jsme vysokou míru proliferace nejen v oblasti vzniku nové lišty, ale také při tvorbě mezer mezi patrovými lištami.

Tato dizertační práce přináší nové významné poznatky o vzniku epitelových derivátů – experimentálně prokazuje výskyt a osud zubních premolárových rudimentů v myší dentici; navrhuje nový model vzniku myší zubní papily a mechanizmus odpovědný za vznik a rozmístění patrových lišt u myši.

Klíčová slova: myš, vývoj, zub, rudiment, zubní mezenchym, papila, folikul, patrová lišta.

Abbreviations

BMP – Bone morphogenetic protein

ED – Embryonic day

EDA – Ectodysplasin A

EGFP – Enhanced green fluorescent protein

FGF – Fibroblast growth factor

IDE – Inner dental epithelium

M1 – First molar

Mesp1Cre/R26R - Cre-recombinase expressed under the control of the Mesp1 promoter as a transgene crossed with the ROSA26 conditional reporter (R26R)

MS – Mesial segment – the anterior premolar rudiment

P - Postnatal

pEK – Primary enamel knot

PH-3 – Phosphohistone-3

R2 – Rudiment 2 – the posterior premolar rudiment

RTK – Receptor tyrosine kinase

SHH – Sonic hedgehog

Spry – Sprouty

Wnt1Cre/R26R - Cre-recombinase expressed under the control of the Wnt1 promoter as a transgene crossed with the ROSA26 conditional reporter (R26R)

WT – Wild type

1. Introduction

1.1 Why teeth?

Teeth are important structures needed for eating and speaking, they are necessary components of our every day life. They are also an integral part of our self-esteem and image. Many types of developmental tooth defects have been described in humans: alteration in tooth size, shape, position and number - from agenesis to supernumerary teeth. The genes implicated in the origin of many of these dental defects have been identified and several mouse models have been created. These mice allow us to work on the particular dental defects, to understand the molecular basis of dental diseases and to find the methods of prospective treatments which could not have been directly investigated and tested on humans (reviewed in Fleischmannová et al. 2008).

Currently, the medical approaches have reached a great grade, ensuring the repair and improvement of the dentition in many inherent dental diseases. However teeth can become a serious problem during later stages of human life. Therefore a special interest has been placed on the possibility of bioengineering a tooth, which could replace a missing or damaged tooth. This new approach is becoming a reality, aided by our increasing understanding of embryonic development, molecular mechanisms, stem cell biology and tissue engineering. Recently a fully functional biological tooth replacement was achieved in an adult mouse by transplantation of a bio-engineered tooth germ into the alveolus of an extracted tooth (Ikeda et al. 2009).

The developing dentition is also a fascinating field to investigate mechanisms of organ system development. During embryonic odontogenesis we can study not only developing dentition as a whole organ system but also its interactions with the surrounding tissues at the same time. Knowledge of tooth development can also help in studies of other epithelial appendages, which go through a similar process of initiation and bud formation, such as hair, salivary, sweat and mammary glands.

1.2 Embryonic tooth development in mouse

Teeth belong to epithelial skin appendages together with hair, palatal rugae, glands (such as salivary, sweat and mammary). These derivates have similar initial features in their development. Teeth develop from dental epithelium and neural crest-derived mesenchyme and their development is driven by reciprocal interactions. During mouse embryonic development the dental epithelium thickens at embryonic day (ED) 11.5, invaginates into the surrounding condensing dental mesenchyme and forms a tooth bud (ED13.5). The dental epithelium grows and starts to fold to produce firstly a cap (ED14.5), and then later a bell (ED16.5) shaped enamel organ, with the mesenchymal dental papilla forming in between the extending epithelial cervical loops (see the Fig. 1A-D for the detailed tooth stages and how they develop *in vitro*). After apposition of hard tissues (dentin, enamel and cement) (Fig. 1F), the dental papilla becomes a pulp with blood vessel and nerve supply (Fig. 1F).

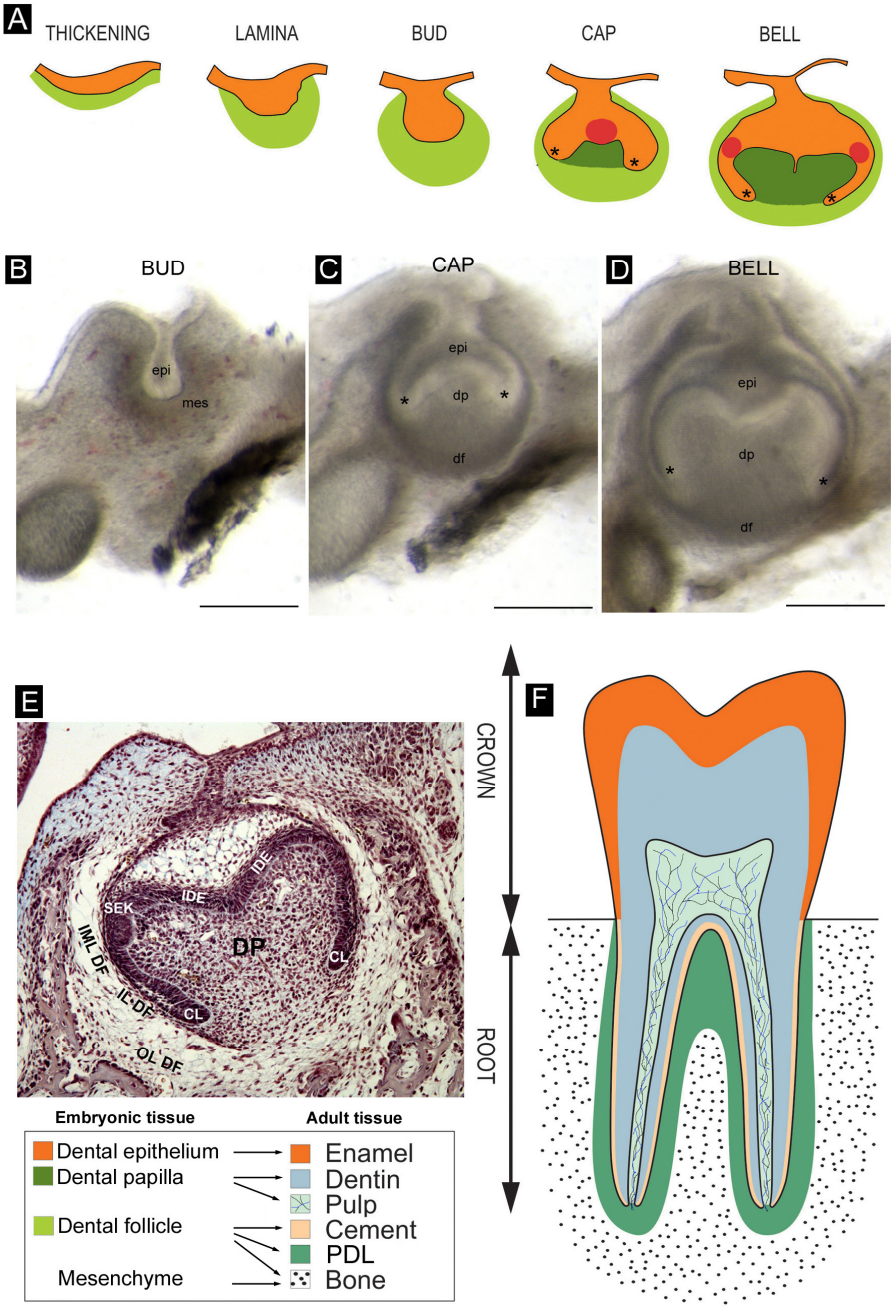

Fig.1 Stages of tooth development in mouse in a scheme, histological section and *in vitro* culture. (A) A schematic of tooth development on frontal sections. Dental epithelium in orange, epithelial enamel knots in red. Dental mesenchyme in green: Light green shows dental mesenchyme at the first stages – thickening, lamina and bud, from cap stage onwards, the light green shows mesenchymal dental follicle. Dental papilla is in dark green and **(A,C,D)** stars indicate cervical loops. The particular stages are called according to the shape of the dental epithelium on frontal sections. **(B,C,D)** Slices of a molar tooth germ cultured *in vitro*. **(B)** A bud stage, day 0. **(C)** A cap stage, the same molar tooth germ after 2 days *in vitro* culture. **(D)** A bell stage, the same molar tooth germ after 4 days *in vitro* culture. Df – dental follicle, dp – dental papilla, epi – epithelium, mes – mesenchyme. Scale shows 250µm. **(E)** Histological frontal section of the mouse M1 at ED 16.5. CL – cervical loops, DP – dental papilla, IDE – inner dental epithelium, IL DF – inner layer of dental follicle, IML DF – intermediate layer of dental follicle, OL DF – outer layer of dental follicle, SEK – secondary enamel knot. **(F)** A scheme of an adult tooth. PDL – Periodontal ligament.

1.2.1 Signalling centers of tooth development

Since the discovery of the organizing activity of the blastopore lip by Spemann and Mangold in 1924, many more types of organizing tissue, regulating the formation and shape of organs, have been discovered in vertebrates. The dental organ has been proposed to have its own signalling centers and the first most prominent is called the primary enamel knot (pEK) (Jernvall et al. 1994). The enamel knot has been known as a structure within molar tooth germs for more than 100 years, and is represented by a group of tightly packed epithelial cells in the middle of the dental cap (Fig. 1A). The role of the enamel knot is to control tooth morphogenesis (proposed already by Butler 1956), from its position in between the epithelial cervical loops and underlying dental mesenchyme, by promoting cell proliferation while the structure itself remains specifically non-proliferative (Jernvall et al. 1994, Vaahtokari et al. 1996). At the molecular level, the pEK is specified by the expression a number of signalling factors, including Shh, Fgf4, 9, Bmp2, 4 and 7 (reviewed in Thesleff and Sharpe 1997). Shh expression before the first molar (M1) cap formation, at the earlier stages of mouse odontogenesis (Dassule and McMahon 1998), has been imputed to the successive development of the M1 (e.g. Jernvall and Thesleff 2000). Contrary to this, it has been suggested, that this early expression pattern corresponds to the signalling centers of the rudimental tooth primordia of lost premolars, which transiently develop in mouse dentition, and not of the developing M1 (Peterková et al. 2000; see Chapter 1.3).

Secondary enamel knots are formed at the position of the future tooth cusps (Fig. 1A,E) and drive cusp formation at the early bell stage, ED16 (Jernvall et al. 1994). They similarly express Shh and Fgf4 as the pEK (Jernvall et al. 1994; Vaahtokari et al. 1996). However, at this stage, the Shh expression is not only restricted to the enamel knots, but is associated with the whole of the inner dental epithelium (IDE) (Bitgood and McMahon 1995). It is assumed that the signalling role of the primary and secondary enamel knot centers is terminated by apoptosis of the signalling cells (Vaahtokari et al. 1996).

1.3 Diastemal rudiments – lost premolars in mouse dentition

The mouse has a reduced dentition that consists of one set of teeth. In each jaw quadrant, we can find one incisor separated from three molars by a toothless gap called the diastema, where incisor, canine, and premolar teeth are present in other species. In early rodents in the

mouse lineage, in some extant rodents and in rabbits, premolars develop anterior to molars (Luckett and Hartenberger 1985). Despite the lack of premolars in the adult mouse dentition, there is morphological evidence for large bud-like structures that develop earlier, anterior to the upper and lower M1, and which have been interpreted as rudimental (vestigial) premolar buds (Peterková et al. 2000). Therefore an understanding of the embryonic development of the diastema region of both - the lower and upper jaws is interesting from an evolutionary aspect. These rudimental buds represent prominent tooth structures at ED12.5 and ED13.5. This is important as they can be easily mistaken for the early stages of M1 development (Fig. 2). The epithelium of these rudiments then gradually regresses by apoptosis (Peterková et al. 1998; Viriot et al. 2000). In the lower jaw these rudimental buds are called MS and R2 and they represent prominent tooth structures at ED12 (MS rudiment) and at ED13 (R2 rudiment). The lower mouse M1 becomes the prominent structure posteriorly by ED14 and morphological data suggests that the repressed R2 is integrated into the anterior part of the M1 cap (Fig. 2) (Viriot et al. 2000).

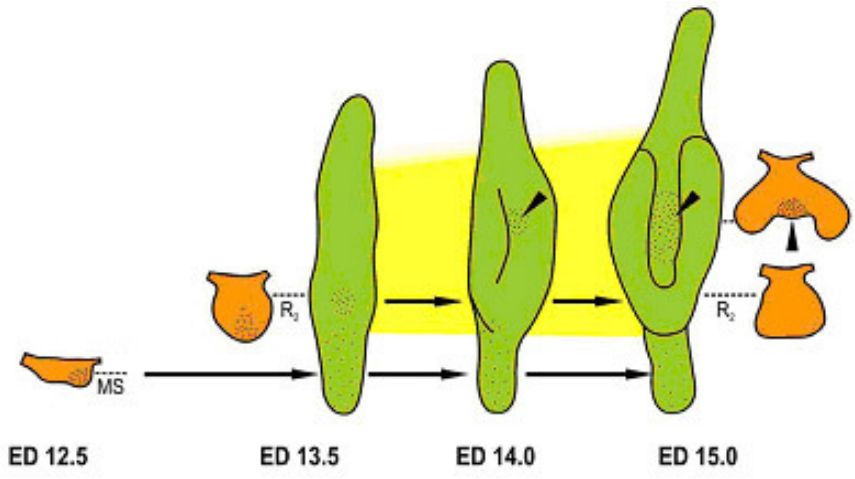

Fig.2 Rudimental tooth germs and the developing M1. Frontal sections in orange, the dental epithelium developing in cheek region in green. Region corresponding to the first molar development highlighted in yellow. Black dots indicate apoptosis, the black arrowheads point out primary enamel knot. Black arrows show the localization of the rudiments, dotted lines the corresponding frontal section. Adapted from Peterková et al. 2000.

1.3.1 Supernumerary teeth in mouse dentition

In some mutant mice there is a supernumerary tooth found anteriorly to the M1 in the originally toothless diastema. These cases are results of molecular disturbances caused by various gene alterations. Here are listed a few examples of mutant or transgenic mice, which exhibit an abnormal supernumerary tooth:

- Tabby, downless or sleek mutant mice (Gruneberg 1966; Sofaer 1969), with defects in Ectodysplasin A (EDA) signalling pathway (e.g. Elomaa et al. 2001) but surprisingly also the transgenic mice over-expressing the EDA receptor EDAR (Tucker et al. 2004) or the EDA protein itself (Kangas et al. 2004).
- Ectodin/WISE deficient mice (Kassai et al. 2005; Ahn et al. 2010).
- Mice homozygous for a null allele of Spry2 or Spry4 (Klein et al., 2006).
- Mutation of Lrp4 and Wise mediating BMP and Wnt canonical signalling (Ohazama et al. 2008)
- Mutation in primary cilia protein Polaris mediating Shh signalling (Zhang et al. 2003; Ohazama et al. 2009).

The supernumerary tooth has been considered as an extra molar (Mustonen et al. 2004; Pispa et al. 2004; Kasai et al. 2005) or related to the lost premolar (Kangas et al. 2004; Tucker et al. 2004), or to the continuous development of a diastemal rudimental bud (Klein et al. 2006). Some authors explicitly proposed the supernumerary tooth takes its origin from the rudimental premolar structures in mouse diastema and thus could represent an atavistic premolar from the mouse ancestor (Peterková 1983; Peterková et al. 2002, 2005; Ohazama et al. 2008, 2009).

Development of supernumerary teeth has become an intensively studied topic over the last decade (reviewed e.g. in Cobourne and Sharpe 2010; Wang and Fan 2011) and brings new insights into the evolutionary aspects of regression and revitalization of tooth development.

1.3.1.1 Sprouty2 mutant mice and supernumerary tooth formation

Sprouty (Spry) genes encode antagonists of the Fibroblast growth factor (FGF) signalling pathway and of other receptor-tyrosine kinase (RTK) ligands (Minowada et al. 1999). In WT mice, Spry2 is expressed in the dental epithelium and mice carrying mutation in this gene develop a supernumerary tooth in front of the lower M1. This is a result of lack of FGF antagonists and up-regulation of FGF signalling in diastema region, which leads to the abnormal survival and development of diastemal tooth buds (Klein et al., 2006).

1.4 The first molar in mouse dentition - dental mesenchyme development

Morphological data have documented that during the first phase of mouse odontogenesis, the prominent tooth germs in the cheek region of a jaw are only rudimental ones, presumably corresponding to the lost premolars as described in Chapter 1.3. However from ED14, the M1 becomes the most prominent structure in the posterior jaw, located in the cheek region. At ED14.5, the tooth bud transforms into a dental cap (Fig. 1A-D): epithelial cervical loops in-grow into the underlying condensed mesenchyme and start to define the region of mesenchymal dental papilla, which is found between these protruding epithelial cervical loops. During bell stage, the cervical loops continue to grow into the surrounding dental mesenchyme with a more distinct dental papilla (Fig. 1A-E). Most of the cells of the dental mesenchyme are of neural crest cell origin as it has been shown, by using Wnt1cre/R26R reporter mice, with some non-neural crest cells contribution (Chai et al. 2000). Dental mesenchyme gives rise to the dental papilla, which later becomes the dental pulp with blood vessel and nerve supply (Fig. 1F), and also to the dental follicle (Fig. 1A,E), which later forms the periodontium: cementoblasts, periodontal ligament and alveolar bone (Fig. 1F) (Ten Cate et al. 1971; Yoshikawa & Kollar 1981; Palmer & Lumsden 1987; Diep et al. 2009). At the bell stage of tooth development, the dental follicle is clearly subdivided into 3 layers – the inner, outer and intermediate layer (Fig. 1E). It is thought that the dental papilla mesenchyme contributes to the inner layer of the dental follicle during the bell stage of odontogenesis (Yoshikawa & Kollar 1981; Palmer & Lumsden 1987; Osborn and Price 1988). The dental papilla itself is assumed to originate passively – from the condensed mesenchyme around the tooth germ captured in between the elongating cervical loops (as schematized for example in bite-it.helsinki.fi; Aberg et al. 2004; Tucker and Sharpe 2004; Fig. 3). However a recent study highlighted the dynamics of the dental mesenchyme located by the sides of epithelial bud, contributing to the dental follicle and alveolar bone formation (Diep et al. 2009).

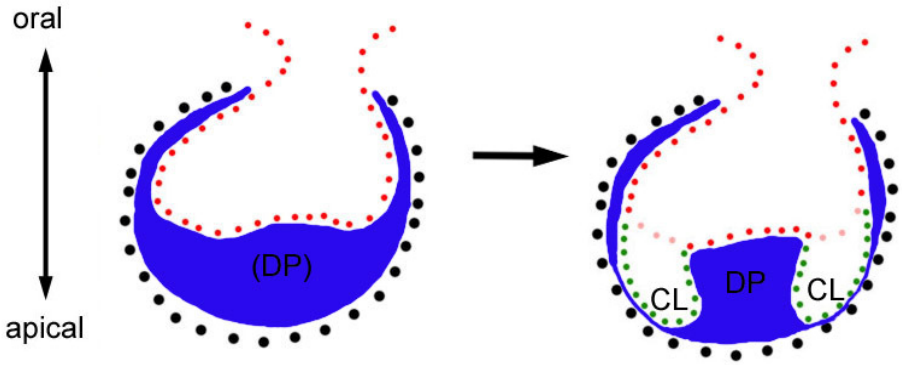

Fig. 3 Assumed mechanism of dental papilla origin. The condensed dental mesenchyme is shown in blue (and black dotted outline). The outline of dental epithelium of lower M1 tooth germ is in red and green. The epithelial cervical loops (in dotted green) extend into the condensed dental mesenchyme (blue) and the enfolded mesenchymal cell population becomes the dental papilla (DP). CL - cervical loop, DP - dental papilla.

1.5 Palatal rugae development

Development of the secondary palate is a critical process in craniofacial embryogenesis. The palatal shelves elevate and fuse during embryonic development, thus separating the nasal from the oral cavities. Failure of this process is responsible for cleft palate, one of the most common birth defects in human. Development of the palate has therefore been extensively studied in the last few decades (reviewed in Gritli-Linde 2007).

Palatal rugae are transversal ridges present on the hard palate and together with the teeth and the tongue, the rugae participate in mastication by helping to sense, hold and mash the food (reviewed in Peterková et al. 1987). Embryonic rugae have long been suspected to help palatal shelve elevation and secondary palate formation, therefore playing a vital embryonic role as well as a later masticatory one (Luke 1984).

Laboratory mice have usually 8 to 9 sets of regularly arranged rugae (Fig. 4). The beginning of rugae development in mouse occurs at ED11.5, when the epithelium thickens and, together with the adjacent mesenchymal condensation, forms a ruga primordium. The next step of ruga development is protrusion of the thickened epithelium into the oral cavity as a convex bud – the primitive ruga. The primitive rugae appear subsequently over time, and by ED15.5 the last formed ruga is present in the palate. At ED17.5, the rugae start to keratinize and form the definitive rugae of the hard palate (Peterková et al. 1987).

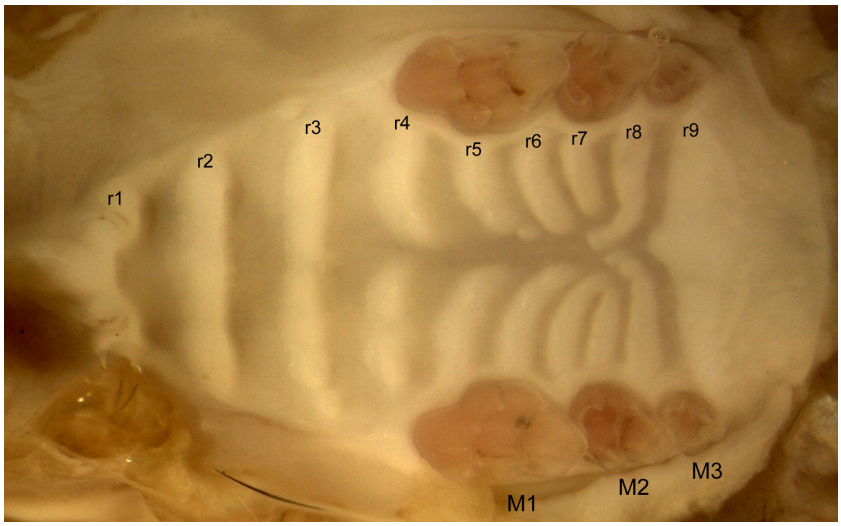

Fig. 4 Upper jaw and palate of WT (CD1) mouse. Palatal rugae marked as r1-r9 according to their antero-posterior localization on the hard palate. M1 - first molar, M2 - second molar, M3 - third molar.

1.5.1 Developmental similarities between tooth and palatal ruga patterning

Ruga morphogenesis shares many similarities with all epithelial appendages during early development – the epithelium thickens and the underlying mesenchyme condenses. Later ruga development differs in the direction of the further growth, and in contrast to invagination of the tooth and hair epithelium, the ruga protrudes (evaginates) in the oral cavity more similarly to feather and scale development (Peterková et al. 1987; reviewed in Mikkola 2007), or to some reptilian tooth development (i.e. alligator) (Westergaard and Ferguson 1990).

Importantly, the palatal rugae and maxillary dentition originate in mouse embryos from a common epithelial thickening on the oral maxillary surface, highlighting the common developmental origin of rugae and teeth (Peterková 1985).

From the molecular view, the tooth, hair, feather and scale early morphogenesis involves common signalling pathways regulating their development (reviewed in Chuong and Noveen 2000; Mikkola 2007). There are also key common players of epithelial appendage regulation involved in rugae formation, including Shh expression, which persists during most stages of their development (Rice et al. 2006) in contrast to teeth (Dassule and McMahon 1998). The rugae pattern is disrupted in EDA mutant mice (Charles et al. 2007) and rugae are lost in Fgf10 mutant mice (Rice et al. 2004). Therefore the knowledge of rugae development can significantly help us in understanding the odontogenesis and other epithelial appendages.

1.6 Overview of the literary data and open questions for the PhD project

Teeth and palatal rugae are both important parts of craniofacial development for various reasons and share common developmental processes. Therefore I will be studying the tissue dynamics during their origin and further embryonic development in mouse.

Based on morphological data, the existence of rudimental structures in mouse embryonic dentition has been documented many times and the most prominent rudiments transiently occur in front of the M1 (Peterková 1983; Peterková et al. 1995, 1996, 2002, 2006; Lesot et al. 1998; Viriot et al. 2000). However, the development of these rudiments has not yet been supported by an experimental or molecular study. That is probably why the large tooth buds appearing first in the cheek region at the beginning of mouse odontogenesis, i.e. before ED14.0 (Fig. 2) have still been generally considered as the developing M1 (e.g. http://bite-it.helsinki.fi/). To address this gap in our knowledge, fate mapping the rudimental tooth buds, localization of Shh expressing signalling centers, and analysis of the spatio-temporal differences in cell proliferation pattern, are key experiments to allow an understanding of the initially progressive development and later growth arrest of the rudimental tooth germs in front of the mouse M1.

The origin of the supernumerary tooth in front of the M1 in some mutant mice from a rudimental diastemal tooth primordium (Peterková 1983) has been supported by morphological (Peterková et al. 2002, 2005, 2006) and molecular (Klein et al. 2006; Ohazama et al. 2008) data. However the presumed revival of the diastemal (premolar) rudiment has not been documented at the level of the morphogenetic mechanisms such as the role of changes in cell proliferation and apoptosis.

From ED14.0 onwards, the M1 is the prominent structure in the mouse embryonic cheek region. At this stage, the mesenchymal dental papilla starts to form. The condensed dental mesenchyme surrounding the epithelial tooth bud and cap is considered as a passive tissue, which is encompassed by the in-growing cervical loops of active epithelium (Fig. 3). However a recent study has shown that the dental mesenchyme on either side of the epithelial bud is a dynamic cell population contributing to the dental follicle and later to alveolar bone formation (Diep et al. 2009). Therefore the fate of the cells of the original dental mesenchymal condensation, their contribution to the dental papilla and follicle and the cell proliferation during the dental papilla formation is an important area for further in-depth investigation.

The mouse dental mesenchyme has been shown to be of neural crest origin (Chai et al. 2000). Interestingly, these authors also reported presence of some non-neural crest cells in the dental mesenchyme from the bud stage of tooth development onwards and their origin is unknown. Together with the investigation of the fate of the dental mesenchyme during the dental papilla formation, it seems necessary to also explore the identity of these non-neural crest cells in the mouse dental papilla and reveal if and when any mesoderm-derived cells contribute to the formation of the dental papilla.

Palatal rugae which have common developmental origin with teeth are parallel transverse ridges present on the hard palate. Their development provides a new and simple model for studying mechanisms of origin and development of epithelial appendages such as teeth and can also bring new insights into the general principles of morphogenesis and palatogenesis. Rugae development is an interesting sequential process, which has not yet been extensively studied. There are many basic questions that need to be addressed, such as how exactly the palatal rugae appear in time and space during palate development and what is the role of cell proliferation in this process.

2. Aims of the thesis

2.1 Development of the rudimental cheek tooth primordia in mouse mandible and the role of cell proliferation

To clarify whether the prominent bud-like structures detected in WT mouse mandible at embryonic day 12 or 13 reflect different rudimental tooth germs in front of the M1 or successive stages of M1 development.
Publication 1.

To verify the hypothesis that the supernumerary tooth observed in Sprouty2 mutant mice results from the revitalization of a rudimental tooth primordium in front of the lower M1 including increase of cell proliferation and/or decrease of apoptosis in the rudimental dental epithelium.
Publication 2.

2.2 Origin of the dental papilla of the first mouse molar

To reveal the origin of the mesenchymal dental papilla of the mouse lower M1, the dynamics and cell proliferation of the dental mesenchyme during dental papilla formation and to investigate the developmental relationships between the condensed dental mesenchyme, dental papilla and follicle.
Publication 3.

To explore the contribution of mesoderm-derived cells as an influx of non-neural crest cells to the dental papilla of the mouse lower M1, the exact timing and the cell identity.
Publication 4.

2.3 Palatal rugae origin, spacing and the role of cell proliferation

To establish a precise temporal sequence of origin of the mouse palatal rugae (which have a common developmental origin with teeth) and the role of epithelial cell proliferation during rugae appearance and spacing.
Publication 5.

3. Results and Discussion

Most of the results described in this chapter have been published in peer-reviewed journals and this chapter contains a concise summary of these publications. The individual contributions of Michaela Rothová to these publications are listed in Chapter 6: List of publications.

3.1 Development of the rudimental cheek tooth primordia in mouse mandible and the role of cell proliferation

Previous morphological data have suggested that development of rudimental tooth primordia in the mouse early embryonic dentition has been mistaken for the M1 development for many years. We tried to bring the first experimental proof of the transient existence of the rudimental diastemal tooth primordia located in front of the M1 and to determine their developmental dynamics and fate in the normal mice and in the mice with a supernumerary tooth in front of the M1.

3.1.1 The rudimental tooth buds have their own signalling centers

To investigate the dynamics of tooth development in the cheek region of embryonic mandible, we first assessed Shh expression, which is a marker of tooth signalling centers during odontogenesis (Thesleff et al. 2001), at closely staged embryos (Peterka et al. 2002) of Shh-EGFP mice (Harfe et al. 2004), in which EGFP (Enhanced green fluorescent protein) is inserted at the Shh locus.

We were able to distinguish 3 separate domains of strong Shh expression, which appeared in the antero-posterior sequence in the mandible over time (data shown in *Publication 1*). This pattern was consistent with the sequentially developing morphological structures of MS, R2, and M1 (Fig. 2). The finding of a series of three Shh-signalling centers, which co-localized with three successive buds (MS, R2, and M1, respectively), strongly contrasts with the classical interpretation of Shh expression in the cheek region of mouse mandible, which is generally attributed to successive stages of M1 development during ED12 to 14 (e.g. Jernvall and Thesleff 2000).

Interestingly, other markers specific for the M1 signalling center – the pEK, such as the activation of the Wnt pathway reported by TOPGAL transgene activity and expression of Edar, p21, Bmp4 and Fgf4 (Jernvall et al. 1994; Tucker et al. 2000; Liu et al. 2008), were also present in these three distinct signalling centers related to the antemolar rudiments and the M1 (data shown in *Publication 1*). This finding suggests that similar gene networks are reiteratively deployed in the development of each MS, R2, and M1, and further strengthens the view that MS and R2 are integral components of successive development of mouse cheek teeth series as rudiments of an ancestral dentition.

3.1.2 Rudimental tooth bud R2 is integrated into M1

To reveal the fate of the rudimental tooth buds, the MS or R2 buds of Shh-EGFP mandibles were labelled at the signalling center with lipophilic dye DiI (at ED 12.7 or 13.3, respectively), and then traced by time-lapse microscopy *in vitro*. In both cases, after the *in vitro* culture the label was localized anteriorly to the rising EGFP signal of the M1 pEK. These results confirmed that the signalling centers of the MS, R2, and M1 are topographically distinct serial structures. However, when the R2-labelled mandibles were cultured longer, the EGFP signal of the M1 pEK spread anteriorly to overlap with the R2 label (data shown in *Publication 1*). Thus, the M1 Shh signalling center (pEK) is initiated in a field more posterior to the R2, which is then integrated into the M1, confirming previous suggestions (see the Chapter 1.3).

3.1.3 Developmental arrest of the rudimental tooth bud R2 – A change of cell proliferation pattern at ED13.5

Our molecular data documented that each of the rudimental buds (MS and R2) has its own signalling centre and the R2 rudiment is incorporated into the M1. It is assumed that the rudimental buds pass through a period of a progressive growth before their growth arrests in front of the rapidly growing M1 from ED13.5 (Viriot et al. 2000; Fig. 2). Therefore we investigated cell proliferation in the R2 bud versus molar region in developmentally less and more advanced mouse embryos at ED13.5, i.e. before and after the presumed growth arrest of the R2. In each case precise stages were specified by embryonic weight (Peterka et al. 2002). To detect the cell proliferation we used Ki67 antibody as a proliferation marker.

We determined two groups of ED13.5 mouse embryos: developmentally less advanced – body weight 150-160 mg - and developmentally more advanced – embryonic body weight 240-250 mg. The proliferation pattern differed between the R2 and M1 regions in both - the dental epithelium and mesenchyme. In the less advanced ED13.5 embryos, we detected high proliferation labelling in the dental epithelium and mesenchyme in the anterior part of the dental epithelium in the cheek region (Fig. 5A,B), which indicated progressive growth of the diastemal R2 rudiment. On frontal sections there was a wide epithelial R2 bud with a specifically located non-proliferating region at the tip (Fig. 5B), where morphological structure of the R2 enamel knot is present (Peterková et al. 2002) and where the rudimental signalling center was found at ED13.5, as described in the Chapter 3.1.1. In the posterior direction, the proliferation labelling decreased (Fig. 5A,C).

In the more advanced ED13.5 embryos, we recorded a change of cell proliferation pattern. In comparison to the less advanced embryos, the anterior epithelium and mesenchyme of the R2 exhibited a marked reduction of the proliferation labelling (Fig. 5D,E). In contrast the M1 started to grow and became prominent due to the increase of cell proliferation in the dental epithelium and the adjacent condensed mesenchyme (Fig. 5D,F). The most posterior part of the dental epithelium and mesenchyme still exhibited low proliferation labelling (Fig. 5D). We also documented a non-proliferative domain in the dental epithelium (Fig. 5D), which most probably corresponds to the pEK of the M1, where absence of proliferation, and also expression of Shh and other signalling molecules such as Fgf 4 and 9 have been found (Jernvall et al. 1994; Vaahtokari et al. 1996), but none of the FGF receptors (Kettunen et al. 1998). The dental epithelium and mesenchyme adjacent to this non-proliferative domain exhibited stronger proliferation labelling than the rest of the tooth primordium (Fig. 5D). These data are in agreement with the previously described findings that the Shh expression has been correlated with an increase in cell proliferation in dental epithelium and surrounding mesenchyme (Hardcastle et al. 1998, Cobourne et al. 2001) and a similar role is also attributed to the FGF molecules (Kettunen et al. 1998).

The detection of the proliferation marker Ki67 revealed the developmental dynamics of the dental epithelium and the growth arrest of the R2 rudiment at ED13.5. We observed the onset of the regression of the R2 rudiment by a marked decrease of this cell proliferation marker in the R2 region compared to the emerging M1 in the posterior area. The regression of the R2

could be caused for example by a suppression of FGF molecules, which are known as strong mitogens, by members of the Sprouty family – the RTK signalling inhibitors present in the diastemal region (Klein et al. 2006).

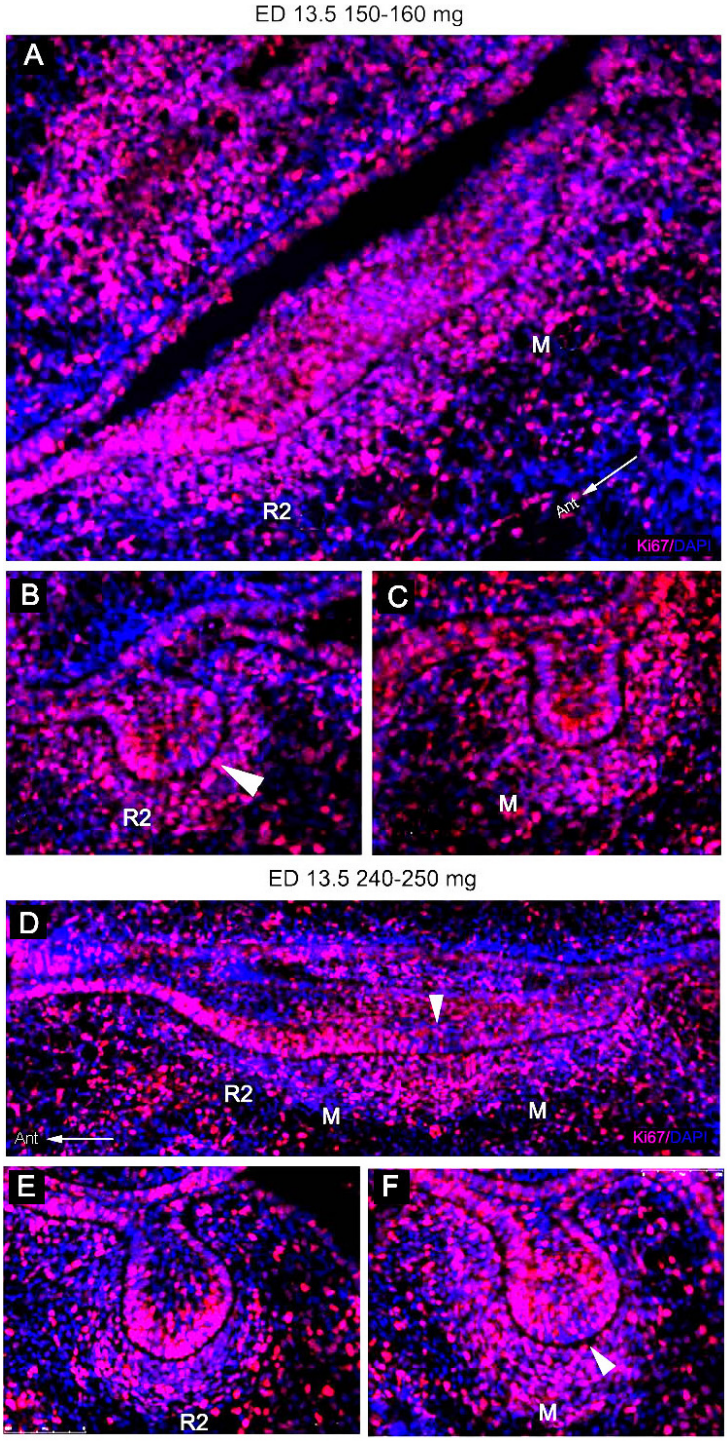

Fig. 5 Detection of cell proliferation during tooth development in the lower cheek region of ED13.5 mouse embryos. **(A,B,C)** Developmentally less advanced embryos at ED13.5 – body weight 150-160mg. **(A)** Sagittal section. The R2 located in the anterior part of the dental epithelium and mesenchyme exhibits high proliferation labelling compared to the molar (M) region. **(B,C)** Frontal sections of **(B)** R2, **(C)** M1 areas. **(B)** The white arrowhead points to a non-proliferating region at the tip of the R2 bud corresponding to the R2 signalling center. **(D,E,F)** Developmentally more advanced embryos at ED13.5 – body weight 240-250mg. **(D)** Sagittal section. The anterior part corresponding to the R2 exhibits marked lower rate of proliferation labelling then the adjacent molar region. The white arrowhead points out a non-proliferating region in the more posterior area - the pEK of the M1. **(E,F)** Frontal sections. **(E)** Proliferating cells are located only at the tip of the R2 tooth bud. The rest of the R2 epithelium and also the dental mesenchyme exhibit low proliferation labelling. **(F)** Both, the dental epithelium and mesenchyme of the M1 strongly proliferate. The white arrowhead points out a non-proliferating region of the pEK. Nuclei in blue (DAPI), proliferating cells in pink (Ki67).

3.1.4 Revitalization of the rudimental R2 tooth bud in Sprouty2 mutants – A role of cell proliferation and comparison to apoptosis

As we showed, a change of proliferation pattern and an important decrease of cell proliferation occur in the region of R2 at ED13.5, leading to suppression of the autonomous growth of the rudimental tooth bud, presumably of the lost premolar. It has been demonstrated that apoptosis is also a mechanism involved in the repression of the development of the large rudimental tooth buds during ED12.5 - 13.5 (Peterková et al. 1998; Viriot et al. 2000). Even though the autonomous development of the rudimental primordia is repressed during mouse tooth development, it has been shown, that the autonomy can be revitalized by altering FGF signalling to form a supernumerary tooth in the cheek region of the lower jaw (Klein et al. 2006).

Therefore, we decided to test a hypothesis: The absence of an FGF antagonist prevents the growth arrest by increasing cell proliferation and decreasing apoptosis in the rudimental tooth bud, which results in its abnormal survival and supernumerary tooth formation in Spry2 mutant mouse embryos. We compared the development of the rudimental tooth bud R2 and the molar epithelium in the mandible of Spry2-/- and WT mice at ED13.5 using histological sections, 3D reconstructions and quantitative evaluations of cell proliferation (mitotic index) and apoptosis.

In WT embryos at ED13.5, the cell proliferation was significantly higher in molar region than in the R2 epithelium undergoing growth arrest (Fig. 6). Apoptosis was mainly concentrated in the anterior part of the dental epithelium, where the diastemal rudiment R2 was repressed. Compared to WT embryos, the mitotic index was significantly higher and the apoptosis was significantly lower in the R2 region of Spry2 mutants and these data confirmed our proposed hypothesis (Fig. 6). Therefore the ectopic increase of the FGF signalling in the rudiments region leads to the revitalization of the rudimental tooth bud R2 by increased cell proliferation and decreased apoptosis. Since the rudimental structures in the posterior part of diastema presumably correspond to the lost premolars, the related supernumerary tooth in the mouse mutants may be considered as a premolar re-appearance (Peterková 1983; Peterková et al. 2002).

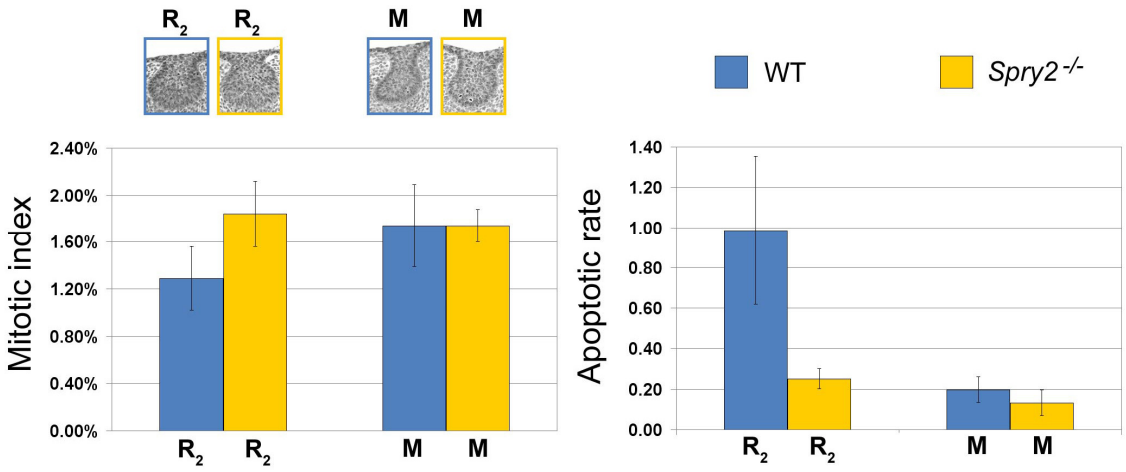

Fig. 6 Comparison of mitotic index and apoptosis between R2 and M1 in WT mice and Spry2 mutant mice. WT in blue, Spry2 mutants in yellow. There is a significant increase of cell proliferation and significant decrease of apoptosis in the R2 of the Spry2 mutants compared to the WT R2 bud. There is no change of proliferation nor apoptosis rate in the molar region of the WT and Spry2 mutants.

Also in other cases of supernumerary tooth formation in mouse dentition, it is possible to distinguish genes responsible for promoting cell proliferation, even though it is necessary to note that the signalling cross talk is a highly complex process. For example Shh expression is known as an important factor regulating cell proliferation (reviewed e.g. in Britto et al. 2000; Chuong et al. 2000). When the SHH signalling is up-regulated in EDA mutant mice (Pummila et al. 2007) and disrupted in Polaris mutant mice (Zhang et al. 2003), formation of a supernumerary tooth is observed. In a similar manner to Spry2 mutants, this supernumerary may also be caused by ectopic high proliferation within the diastemal region. Interestingly, it has also been shown recently, that addition of exogenous FGF8 to the isolated diastemal regions rescues the diastemal tooth development, by promoting cell proliferation and inhibiting apoptosis in the diastemal dental epithelium (Li et al. 2011).

3.2 Origin of the dental papilla of the first mouse molar

To investigate the origin of the dental papilla of the mouse lower first molar, we took advantage of slice culture *in vitro* method, which provides a good technique to visualize the tooth germ during development and to preserve the natural environment of the tooth. We fate mapped the dental papilla development in the mouse lower M1 by vital cell labelling such as DiI; DiO labelling and GFP electroporation, by tissue grafting and lineage tracing (using the Cre-lox transgenic mouse system).

3.2.1 The dynamics of dental mesenchyme, formation of the dental papilla and follicle

The dental mesenchyme that gives rise to dental papilla is often assumed as a passive tissue, which is enfolded by the in-growing cervical loops (Fig. 3).

However, when we tested this generally assumed model of dental papilla development, we revealed that only a restricted mesenchymal cell population gives rise to early dental papilla development. At first, we labelled dental mesenchymal cells at the periphery of the condensed dental mesenchyme in the forming M1 cap at ED14.5 and followed their fate (Fig. 7). In contrast to the generally assumed model of dental papilla origin (Fig. 3), this part of the dental mesenchyme was not encompassed by elongating cervical loops and did not contribute to the dental papilla formation but spread into the inner layer of dental follicle at the dental papilla base (Fig. 7).

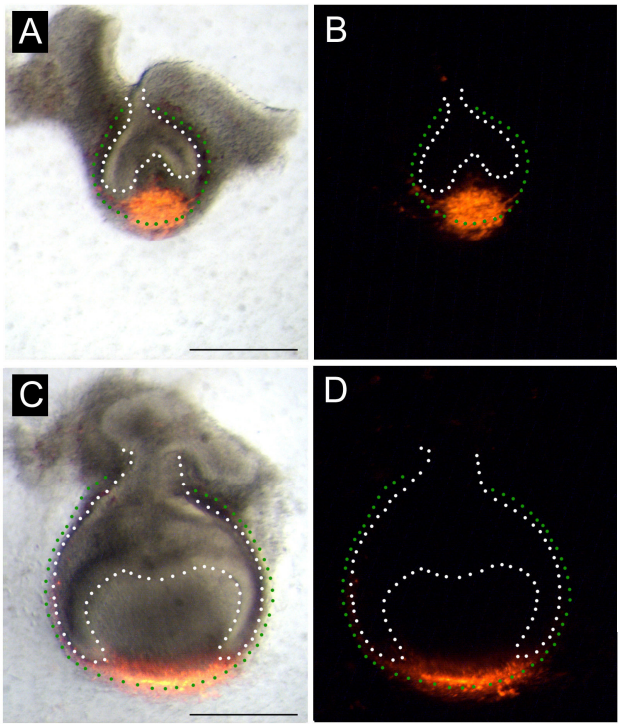

Fig. 7 Condensed dental mesenchyme cell labelling and fate-mapping at ED14.5. (A) A sliced mouse mandible containing M1 at the initiated cap stage at ED14.5. The periphery of the condensed dental mesenchyme (green dotted line) was labelled with DiI (in red). **(B)** Outline of the epithelium of the same M1 under dark field. **(C)** The same molar tooth germ after 2 days of *in vitro* culture. The DiI labelled cells spread into the dental follicle (green dotted line) at the base of dental papilla. **(D)** The same molar tooth under dark field. The dental epithelium is highlighted with a white dotted line. Scale shows 250 µm.

By investigation of the fate of dental mesenchyme further, we revealed, that the mesenchymal cells contributing to the dental papilla formation are located in close proximity to the inner dental epithelium (IDE), in between the cervical loops (Fig. 8). The rest of the dental

mesenchyme contributes to the formation of layers of the dental follicle (Figs. 7 and 8), which will become the periodontium. These new data are in contrast to the generally assumed model of dental papilla origin (as schematized for example in bite-it.helsinki.fi; Aberg et al. 2004; Tucker and Sharpe 2004; compare to Fig. 3) and together with our further investigations bring new insights into the dynamics and origin of the dental papilla (compare to a scheme in Fig. 12).

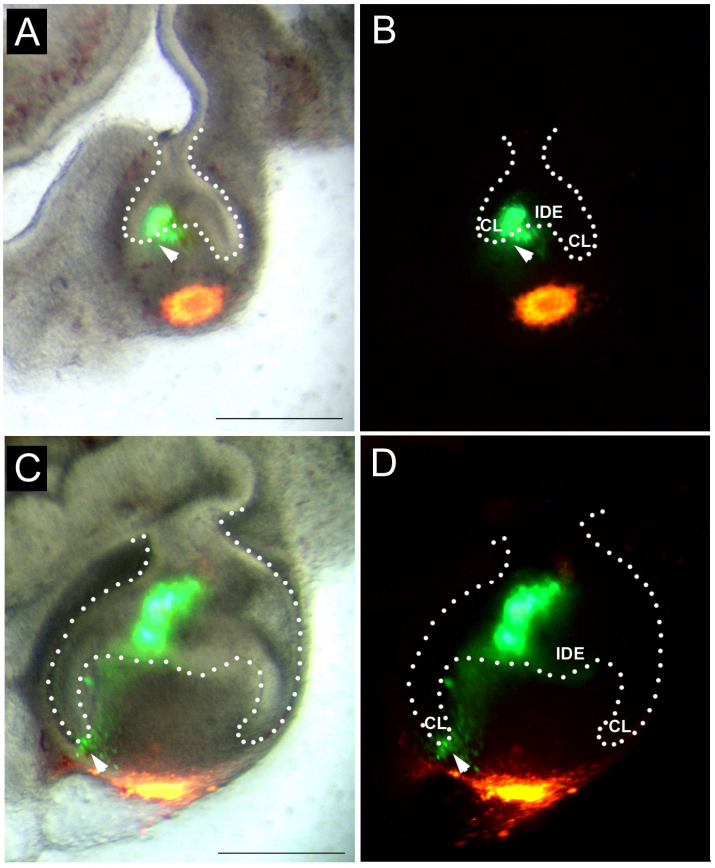

Fig. 8 Cell labelling and fate-mapping of the dental papilla mesenchyme and epithelium at ED14.5.
(A) A sliced mouse mandible containing a tooth germ at cap stage at ED14.5. At day0 a single green spot of DiO labelled the inner dental epithelium and adjacent mesenchymal cells. A cell population at the periphery of the condensed dental mesenchyme was labelled with DiI – in red. **(B)** Outline of dental epithelium of the same molar tooth germ under dark field. **(C)** After 2 days of *in vitro* culture, the single labelled region of mesenchymal cells was spread as a band of green DiO labelled cells spanning across the dental papilla. The labelled dental mesenchyme remained in close contact with the cervical loop (white arrowheads), moving towards the red labelled cells in the forming dental follicle. The DiI labelled cells spread in a layer of the dental follicle adjacent to the dental papilla. The labelled epithelial cells spread within the enamel organ. **(D)** Outline of the same molar tooth under dark field. CL – cervical loop, IDE – inner dental epithelium.
The dental epithelium is highlighted with a white dotted line. Scale shows 250µm.

It has been described that the dental papilla cells themselves contribute to inner layer of dental follicle during late bell stage of odontogenesis (Yoshikawa & Kollar 1981; Palmer & Lumsden 1987, Osborn and Price 1988). However we showed that the dental papilla cells are capable of contributing to the dental follicle as early as cap stage (data shown in *Publication 3*).

By our fate-mapping experiments (by DiI labelling and GFP electroporation), we observed that the dental mesenchyme at ED13.5 is an even more dynamic tissue, than at later stages. Only a small region of labeled cells at ED13.5 was capable of populating the dental papilla and dental follicle around the enamel organ (data shown in *Publication 3*). A comparison

of the capability of the dental mesenchyme between ED13.5 and ED15.5, to contribute to the formation of the various mesenchymal structures (such as dental papilla and follicle), we concluded that the dental papilla mesenchyme at ED15.5 is a more stable and specified mesenchymal cell population then during the first phase of dental cap formation, in the developmentally less advanced embryos, i.e. at ED 13.5 and ED14.5. Therefore the ED15.5 dental papilla is more suitable for studies aiming to target the future dental papilla cells and odontoblasts then any earlier stages.

3.2.2 Detection of cell proliferation during dental papilla formation

To investigate if there are any specific proliferative domains in the dental mesenchyme, which would be responsible for the observed expansion of the dental papilla (Fig. 7), we used two markers of proliferation to map the proliferation pattern during ED14.5 and ED15.5. We performed the double immuno-staining of proliferation marker – Ki67 and mitotic marker PH-3 on serial frontal and sagittal sections of mouse embryonic heads containing lower M1 at ED 14.5 and ED15.5 (Fig. 9).

At both stages, ED14.5 and ED15.5, a high mitotic and proliferative activity was distributed throughout the dental mesenchyme (Fig. 9). At ED14.5 there were no marked differences in cell proliferation between the developing dental papilla and surrounding dental mesenchyme (Fig. 9A-D). At ED15.5 the dental papilla and surrounding dental mesenchyme was also highly proliferative, only cells at the periphery of the surrounding dental mesenchyme exhibited lower cell proliferation labelling (Fig. 9E-H). However we were not able to detect any specifically located domain of high cell proliferation restricted only within the mesenchymal dental papilla, which would be responsible for the dental papilla expansion and which would allow the different regions of the dental mesenchyme (the dental papilla from the follicle) to be distinguished. These results suggest that as the tooth germ enlarges during development, not only the dental papilla expands but also the dental follicle, which is formed from the mesenchyme surrounding the dental papilla and is also capable of high cell proliferation during cap formation. Low cell proliferation was obvious in the very anterior part of the M1 tooth germs, where the premolar rudimental tooth primordium was incorporated (Chapter 1.3 and 3.1.2). Low cell proliferation also exhibited the pEK signalling center as described previously (Jernvall et al. 1994). As mentioned above, this specific epithelial region expresses many

signalling molecules and transcription factors to drive tooth development and epithelium-mesenchyme reciprocal interactions (for review see Jernvall and Thesleff 2000). We propose a model whereby the mesenchymal cells in close proximity to this epithelial signalling center obtain the information sent from the enamel knot and drive dental papilla formation, while cells further away, which do not get the same molecular information, contribute to the dental follicle.

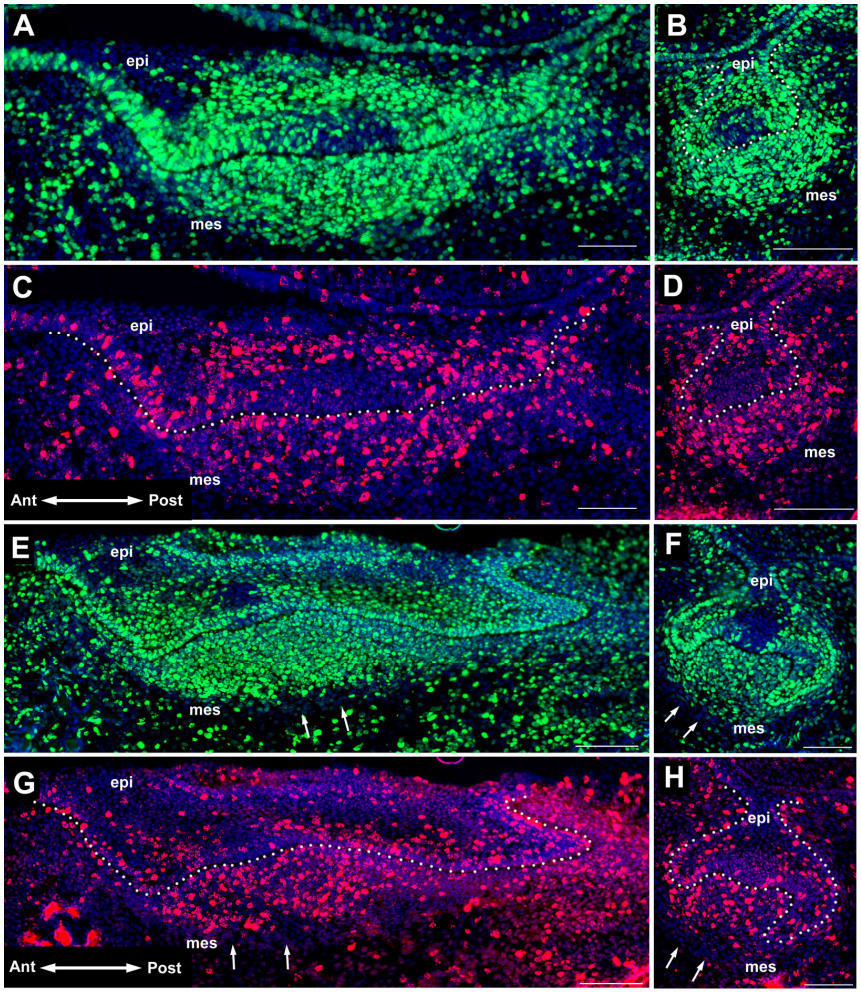

Fig. 9 Detection of cell proliferation on sagittal and frontal sections by using Ki67 and PH-3 antibodies. Immuno-histochemistry of Ki67 antibody – the proliferative marker (in green) and Phosphohistone-3 antibody (PH-3) – the mitotic marker (in red), nuclei (in blue). **(A,C)** Sagittal sections of the M1 at ED14.5 (embryonic body weight 260 mg). There is very high cell proliferation labelling in the condensed dental mesenchyme. The posterior and very anterior mesenchyme exhibits much lower proliferation labelling. **(B,D)** Frontal sections of the M1 at ED14.5 (embryonic body weight 260 mg, the same litter). **(E,G)** Sagittal sections of the M1 at ED15.5 (embryonic body weight 390 - 400 mg). There is high proliferation labelling in the anterior part of the dental papilla. The white arrows point out the periphery of the dental mesenchyme which exhibits low proliferation labelling. **(F,H)** Frontal sections of the M1 at ED15.5 (embryonic body weight 390 - 400 mg, the same litter). The white arrows point out the periphery of the dental mesenchyme which exhibits low proliferation labelling. **(A, B, E,F)** Ki67 immuno-staining. **(C,D,G,H)** PH-3 immuno-staining. Ant – anterior, epi – epithelium, mes – mesenchyme, post – posterior. Scale shows 100μm.

3.2.3 Contribution of mesoderm into the developing dental papilla

The dental mesenchyme has been shown to be of neural crest cell origin, by using a Wnt1cre/R26R reporter mouse (Chai et al. 2000). However, these authors also reported a presence of some non-neural crest cells from the bud stage of tooth development onwards. As the tooth develops to the cap and bell stages, an increasing number of non-neural crest cells were observed in the dental papilla (Chai et al. 2000). The number of non-neural crest cells in the dental papilla can increase as a result of immigration of cells. To test this hypothesis, we fate mapped the cells using the slice *in vitro* culture method and DiI labelling of the mouse lower M1. We documented that cells which contribute to the dental papilla at the bell stage are originally located in the periphery of the condensed dental mesenchyme at the cap stage (data shown in *Publication 4*). Using a Mesp1cre/R26R reporter mouse (Mesp1 as an early marker of mesoderm during gastrulation – Saga et al. 1996) and DiI labelling we determined the labelled cells as of mesodermal origin (data shown in *Publication 4*). To trace the contribution of mesoderm to the tooth germ *in vivo* we looked at histological sections of the Mesp1cre/R26R reporter mouse during various stages of tooth development. Cells of mesodermal origin entered the developing dental papilla at ED15.0 and then further populated the dental papilla (Fig. 10).

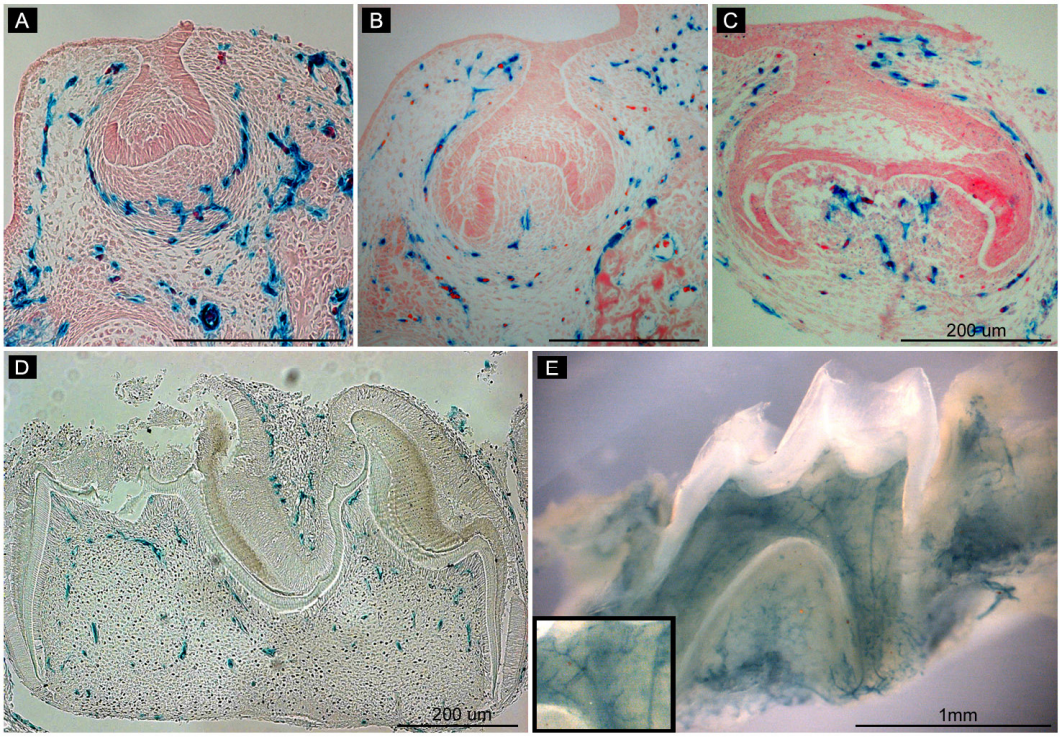

Fig. 10 Contribution of mesoderm-derived cells to mouse molar development. The mesoderm-derived cells are stained with X-gal in blue. **(A)** Frontal section of molar tooth germ of ED14.5 Mesp1cre/R26R mouse embryo. There are no cells of mesodermal origin present in the dental epithelium or in the closely adjacent condensed mesenchyme. **(B)** Frontal section of molar tooth germ of ED15.0 Mesp1cre/R26R embryo. Single mesoderm-derived cells are found in the dental papilla and the forming dental follicle. **(C)** Frontal section of molar tooth germ of ED16.5 Mesp1cre/R26R embryo. The mesoderm-derived cells are present in the dental papilla and the forming dental follicle. **(D)** Sagittal section of the first molar of Mesp1cre/R26R mouse at postnatal day (P) 5. **(E)** Sagittal view of the first molar of Mesp1cre/R26R mouse at P18, showing large numbers of mesoderm-derived cells in the dental pulp and at the roots with high magnification of the capillary network in the frame.

To identify whether the mesoderm-derived cells moving into the dental papilla were endothelial cells (as suspected by Yoshida et al. 2008), CD31 immuno-histochemistry (as a specific endothelial marker) was performed on Mesp1cre/R26R stained sections of the first lower M1. A clear co-localization of CD31 and mesoderm-derived cells was detected in the dental pulp (Fig. 11), documenting the immigration of endothelial cells of mesodermal origin into the dental papilla.

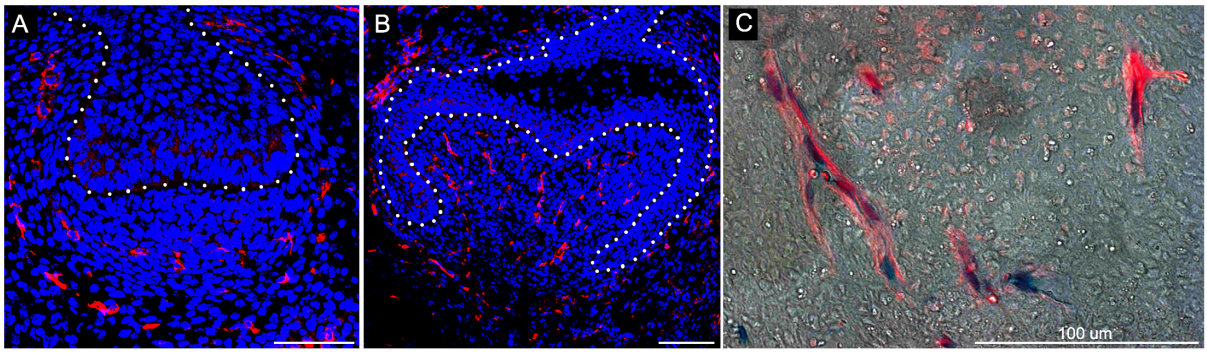

Fig. 11 Endothelial cells in the dental papilla (A,B) Frontal section of WT mouse lower M1 at **(A)** ED14.5 **(B)** ED16.5. CD31 positive endothelial cells are stained in red, nuclei with DAPI in blue. The CD31 pattern corresponds with the Mesp1cre/R26R X-gal stained molar tooth germ shown in Figure 10A,C. **(C)** CD31 staining on Mesp1cre/R26R X-gal stained sections of mouse molar tooth germ at P5. CD31 positive endothelial cells are stained in red and are co-localized with the X-gal stained blue mesoderm-derived cells.

Thus the increase of non-neural crest derived cells in the dental papilla during development is caused by the immigration of mesoderm-derived endothelial cells, which start to infiltrate the dental papilla at ED15.0. No mesoderm-derived cells are present in the condensed dental mesenchyme close to the tooth bud or cap before this time point. Our findings open an interesting question regarding the origin of the non-neural crest cells found at earlier stages of tooth development, thus before ED15.0 (Chai et al. 2000). The Wnt1cre/R26R (Chai et al. 2000) and our Mesp1cre/R26R analysis suggest that these cells appear to be neither of neural crest nor of mesodermal origin. Thus either some other cell population contributes to the tooth mesenchyme at these early stages, or perhaps not all mesoderm or neural crest derived cells are labelled by the above-mentioned reporters.

3.2.4 A new model of dental papilla development and origin

Based on our dental papilla fate mapping experiments, we propose a new model for the origin of the dental papilla (Fig. 12). At ED14.5 there is only a restricted population of dental mesenchymal cells in between the cervical epithelial loops which fill up the dental papilla during development. In the dental follicle layers of ED14.5 mesenchyme, there are mesoderm-derived endothelial cells, which start to immigrate into the dental papilla at ED15.0. Importantly, the dental papilla cells contribute to the inner layer of the dental follicle (Fig. 12).

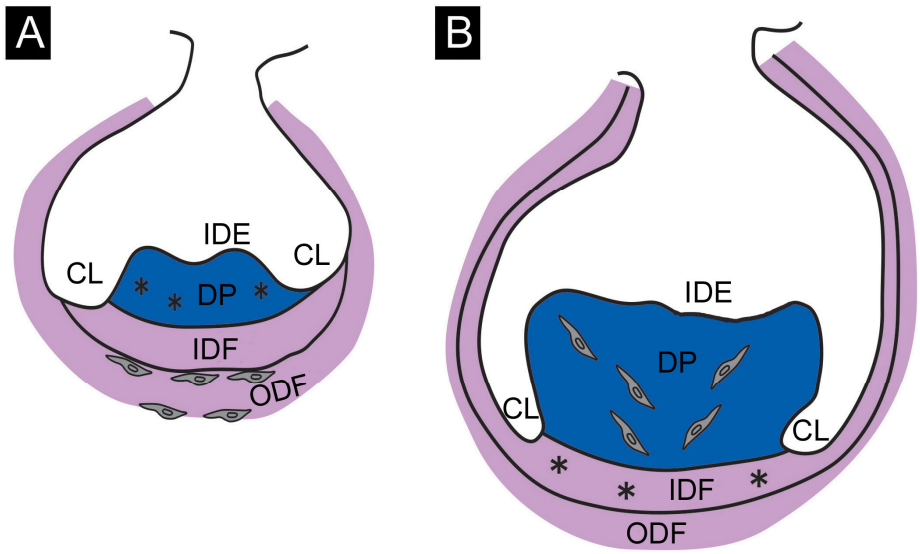

Fig. 12 New model of dental papilla origin. (A) A scheme of a cap staged tooth germ at ED14.5. Dental papilla comprises of only a limited mesenchymal cell population located next to the IDE in between the cervical loops (in blue). The rest of the dental mesenchyme forms the dental follicle with 2 layers distinguishable at the cap stage (in purple). The outer layer of the dental follicle region contains mesoderm-derived endothelial cells (in grey). **(B)** A scheme of a bell staged dental papilla. The blue mesenchymal papilla cells have filled up the space in between the elongating cervical loops and the mesoderm-derived endothelial cells immigrate into this region. Purple mesenchymal follicle cells surround the enamel organ. Some dental papilla cells from the cap staged tooth germ (schematic by * in A) contribute to the inner layer of the dental follicle (schematic by * in B). CL – cervical loop. DP-dental papilla; DF-dental follicle, IDE – inner dental epithelium, IDF – inner layer of dental follicle, ODF – outer layer of dental follicle.

3.3 Palatal rugae origin, spacing and the role of cell proliferation

As with many other epithelial appendages, palatal rugae express Shh at the early stage of their development (Rice et al. 2006). We took the advantage of the fact that the Shh expression persists in rugae during most stages of their development in prenatal mice (Rice et al. 2006) to precisely trace the developmental fate of each ruga. We identified the Shh stripes corresponding to each ruga and followed the development over time accurately according to the embryonic chronological stage specified by body weight (according to Peterka et al. 2002). From very early stages (ED12.0), the sharpest stripe of Shh expression corresponded to ruga 8. The ruga 8 seemed to be the first ruga to appear, but it was very rapidly followed by two stripes corresponding to ruga 2 and ruga 9. From then on, new Shh stripes emerged in between the last formed ruga (the youngest) and the ruga 8. In conclusion, this analysis of Shh expression allowed us to define a temporal sequence for rugae appearance: Ruga number 8 – 2+9 – 1+3 – 4 – 5 – 6 – 7 - 7b (data shown in *Publication 5*).

To analyze the role of epithelial cell proliferation in the process of formation of a new ruga and its spacing, we studied cell proliferation pattern by evaluation of epithelial mitotic index and Ki67 immuno-staining during emergence of ruga 5 and compared to the earlier formed rugae 2, 3, 4 and 8 in mouse embryos at ED13.5. Interestingly, the former (older) rugae (at ED13.5 ruga 2 and 3) exhibited lower epithelial cell proliferation then the regions of non-thickened epithelium (so called gaps) (Fig. 13A). We propose that this high epithelial cell proliferation in the gaps is responsible for the spacing of the rugae and anterior/posterior growth of the palate. A newly forming ruga (ruga 5 at ED13.5) seemed to form from a burst of cell proliferation in the epithelial region immediately posterior to ruga 4 (the last formed ruga at this stage), which exhibited lower epithelial cell proliferation as the former formed rugae 2 and 3 (Fig. 13).

Shh expression was found only later during formation of a primitive ruga when the epithelium is clearly thickened. Interestingly, during tooth development, an epithelial signalling center - the enamel knot - is also formed by non-proliferating and densely packed cells expressing Shh (as described above and reviewed in Thesleff and Sharpe 1997). Moreover, another characteristic of the enamel knot is the presence of apoptotic cells, which was also noted (in ruga 2 and 3 at ED13.5) (Turečková et al. 1996). The Shh expression is generally linked with regulation of cell proliferation (reviewed e.g. in Britto et al. 2000; Chuong et al. 2000) and therefore the presence of Shh expression in rugae might be involved in the stimulation of cell proliferation of the surrounding tissue and the gaps in between rugae, while the formed rugae themselves remain with low-level proliferation in the similar manner as the pEK of the tooth (Jernvall et al. 1994; Vaahtokari et al. 1996; Cobourne et al. 2001). However in contrast to the tooth development, the effects of up/down regulation of SHH signalling in the rugae development have not been studied yet and remain a very interesting question for future studies.

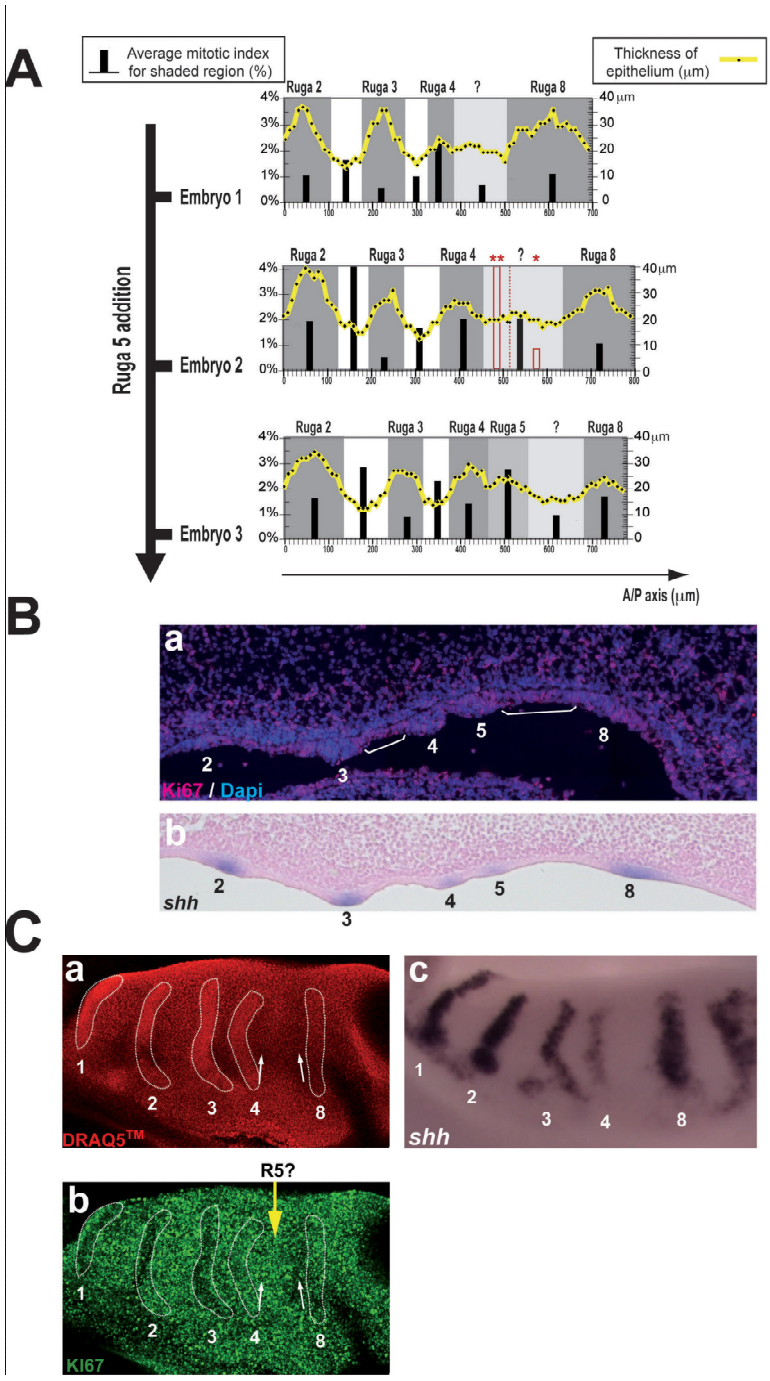

Fig. 13 Epithelial cell proliferation during new ruga formation. (A) Three mouse embryos at three consecutive stages during ruga 5 formation at ED13.5. The individual graphs correlate epithelium thickness - the yellow line with the black points, and mitotic index along the anterior/posterior (A/P) axis of the palatal shelf - black bars. Different regions are shown in grey (ruga), in white (gap), in light grey (region of interest – where the ruga 5 appears). **(B) (a)** Proliferation along the A/P axis of the palatal shelf on sagittal section visualized by the KI67 antibody at ED13.5 (ruga 5 formed) for an embryo of similar weight as the Embryo 3 in **(A)**. Ki67 positive - proliferating cells (in pink), non-proliferating cells in blue (DAPI nuclear staining). White brackets point high cell proliferation of the ruga3-ruga4 gap and the region between the last formed ruga (5) and ruga 8. **(b)** Shh expression (in blue) along the A/P axis of the palatal shelf on sagittal section. **(C)** Dissected palatal shelves at ED13.5 (ruga 5 in formation). **(a)** Nuclear marker Draq5TM. Rugae highlighted with white lines. **(b)** The same palatal shelf immuno-labelled with Ki67 to show proliferation in the epithelium. Note that Ki67 negative cells are found mainly in rugae (especially in more mature rugae 1–3) and in the region immediately anterior to ruga 8 (compare the labelling in the regions pointed by the two white arrows). **(c)** An embryo of similar weight was hybridized with the Shh probe. The large space between ruga 4 and 8 confirmed that the ruga 5 is already developing, as for the embryo 2 in **(A)**.

4. Conclusions

4.1 Development of the rudimental cheek tooth primordia in mouse mandible and the role of cell proliferation

We experimentally proved that the prominent tooth primordia in WT mouse mandible at ED12 or 13 represent rudimental structures corresponding to MS or R2 rudiments, respectively. Each rudiment has its own signalling center. We revealed that one of the mechanisms of the R2 rudiment regression at a bud stage is a marked decrease of epithelial cell proliferation and integration into M1. *Publication 1 and 2*. Interestingly, when Sprouty signalling is altered, the rudiment R2 is revitalized as a result of significant increase of epithelial cell proliferation and decrease of apoptosis to become an independent supernumerary tooth in mouse dentition. *Publication 2*. Therefore we experimentally demonstrated that the prominent tooth primordia at ED12 or 13 should not be misinterpreted as subsequent stages of progressive development of M1 anymore but recognized as distinct rudimental primordia of ancestral teeth. The appropriate tooth primordia identification is essential for correct interpretation of molecular data achieved in the mouse odontogenesis model. *Publication 1 and 2*.

4.2 Origin of the dental papilla of the first mouse molar

We revealed that the mouse condensed dental mesenchyme is a highly dynamic tissue and only a restricted population of mesenchymal cells gives rise to the dental papilla. Therefore we proposed a new model of dental papilla origin, where the cervical loops do not encompass the passive condensed mesenchyme as generally assumed but the epithelial loops grow together with the adjacent active mesenchyme. The remaining dental mesenchyme surrounding the enamel organ and dental papilla gives rise to the dental follicle and both are highly proliferative tissues during ED14.5 and ED15.5. We also showed that the dental papilla cells contribute to the dental follicle formation from as early as the cap stage of mouse molar development. This knowledge is necessary for targeting the progenitors of odontoblasts and dental papilla cells during early stages of mouse odontogenesis. *Publication 3.*

We proved that mesoderm-derived cells represent the contribution of non-neural crest cells into the dental papilla, starting to immigrate at specific time point – at ED15.0. These cells create a network of endothelial cells, forming the blood vessels of the tooth. This brings an important advance in our understanding of how a tooth develops and is particularly relevant to studies which aim to create bioengineered teeth. *Publication 4.*

4.3 Palatal rugae origin, spacing and the role of cell proliferation

We established a temporal sequence of mouse rugae appearance, when the newly forming ruga is always added in between the last formed (the youngest) ruga and the first formed (the oldest) ruga 8, forming the temporal sequence: 8; 2 and 9; 1 and 3; 4; 5; 6; 7. We revealed in a model of ruga 5 formation, that high epithelial cell proliferation is specifically located in the regions in between rugae (gaps) to provide the rugae spacing and that the new ruga is formed as a burst of epithelial cell proliferation right next to the last formed ruga, while the gap in between is formed later. These data importantly broaden our knowledge about epithelial appendages patterning and epithelial-mesenchymal organogenesis and can be useful and translated into development of other epithelial appendages, such as teeth. *Publication 5.*

4.4 General conclusion

Teeth and palatal rugae are epithelial appendages that have common developmental origin and represent important structures in craniofacial development. My PhD study has brought an important insight into the role of cell proliferation and tissue dynamics during origin and development of the epithelial appendage in mouse embryos. We discovered that low cell proliferation plays a major role in the growth arrest of the abortive rudimental tooth germ in mouse dentition. In contrast, high epithelial cell proliferation is involved in both, tooth rudiment revitalization resulting in a supernumerary tooth development and in a new ruga formation and spacing. We also revealed tissue dynamics of the dental mesenchyme and its contribution to the dental papilla and follicle formation. Only a restricted population among the original mesenchymal cell population contributes to the dental papilla formation and from a specific time point, the embryonic day 15.0, the dental papilla is immigrated by mesodermal cells (the prospective endothelium).

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6. List of publications

1) Procházka J, Pantalacci S, Churavá S, Rothová M, Lambert A, Lesot H, Klein O, Peterka M, Laudet V, Peterková R. Patterning by heritage in mouse molar row development. 2010. Proc Natl Acad Sci U S A:107(35):15497-15502.
IF 9.77
Contribution of M.R.:
- cell proliferation study (not included in the final published version)
- a part of Shh in situ hybridizations and their analysis

2) Peterková R, Churavá S, Lesot H, Rothová M, Procházka J, Peterka M, Klein OD. Revitalization of a diastemal tooth primordium in Spry2 null mice results from increased proliferation and decreased apoptosis. 2009. J Exp Zoolog B Mol Dev Evol.;312B(4):292-308.
IF 2.37
Contribution of M.R.:
- a part of the 3D reconstructions; cell proliferation study (Fig. 7)
- data analysis and interpretation
- writing the related parts of the manuscript for the proliferation part

3) Rothová M, Peterková R, Tucker AS. New insight into the origin of the dental papilla. Manuscript submitted to Developmental Biology.
Contribution of M.R.:
- design of the study
- performance of all experiments and figures (except Fig. 1A, which is a contribution of Dr. Abigail Tucker)
- data analysis and interpretation
- writing of the manuscript

4) Rothová M, Feng J, Sharpe PT, Peterková R, Tucker AS. Contribution of mesoderm to the developing dental papilla. 2011. Int J Dev Biol. 55(1):59-64.
IF 2.86
Contribution of M.R.:
- design of the study
- performance of all experiments and figures (except Fig. 5C, which is a contribution of the co-author Jifan Feng)
- data analysis and interpretation
- writing of the manuscript

5) Pantalacci S, Procházka J, Martin A, Rothová M, Lambert A, Bernard L, Charles C, Viriot L, Peterková R, Laudet V. Patterning of palatal rugae through sequential addition reveals an anterior/posterior boundary in palatal development. 2008. BMC Dev Biol.;8:116.
IF 2.78
Contribution of M.R.:
- cell proliferation study (Fig. 3A,B)
- data analysis and interpretation
- writing the related parts of the manuscript on cell proliferation

7. Attachments

The following attachments include the peer-reviewed publications with contribution of Michaela Rothová as listed in Chapter 6.