MASARYK UNIVERSITY

**FACULTY OF SCIENCE**

# Developmental processes involved in the formation of cleft lip and cleft palate

**PhD thesis**

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Bibliografický záznam

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**Vedoucí práce:** Doc. RNDr. Marcela Buchtová, Ph.D.

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Bibliographic entry

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**Keywords:** facial development, cleft lip, cleft palate, secondary palate, primary cilia, veiled chameleon, *Cdk13*, *Tmem107*

Abstrakt

Vývoj obličeje je komplexní proces regulovaný řadou signálních drah. Vlivem vnějších a vnitřních faktorů může dojít k narušení jednotlivých vývojových fází, což následně vede ke vzniku obličejových vad. Mezi nejfrekventovanější vrozené vady obličeje v lidské populaci se řadí rozštěpy rtu a/nebo patra. Odhalení potenciálních etiologických faktorů přispívajících k jejich vzniku je nutným předpokladem, jak jim v budoucnu předcházet.

První část disertační práce se zaměřuje na morfologii sekundárního patra u chameleona jemenského. Během vývoje nedochází u tohoto živočišného druhu k jeho úplnému uzavření, což vede ke vzniku fyziologického rozštěpu. Tento jev je částečně způsoben vlivem nízké proliferace buněk patrových plotének a rovněž na základě měnících se rozměrových parametrů hlavy během vývoje. Snižující se buněčná proliferace v patrových ploténkách je pak způsobena klesající produkcí proteinového ligandu SHH v palatální tkáni. SHH signální dráha zde pravděpodobně hraje roli v medio-laterálním růstu patrových plotének. Rozdíly v růstu a morfologii patrových plotének u chameleona jsou doprovázeny změnami v expresi genů *Msx1*, *Meox2* a *Pax9*, které se významně uplatňují v rostro-kaudálním modelování patra také u savců.

Druhá část práce se soustředí na myší modely prezentující výrazné vývojové vady v kraniofaciální oblasti, které jsou způsobeny mutacemi ve dvou různých genech. V případě prvního modelu se jedná o mutaci v genu *Cdk13* kódujícím enzym protein kinázu CDK13 regulující transkripci a alternativní sestřih. Fenotypový projev mutace v *Cdk13* se u myší liší v závislosti na formě mutace. Mutant nesoucí hypomorfní alelu má méně výrazný fenotyp zahrnující rozštěp patra, kompletní myší knockout má rozsáhlé vady centrální části obličeje včetně rozštěpu rtu a patra. Rovněž u lidí způsobuje mutace tohoto genu četné vývojové vady, včetně kraniofaciálních malformací.

Myší model s mutací v genu *Tmem107* slouží jako model tří syndromů popsaných u lidí. Jedinci postižení některou z těchto poruch vykazují typický kraniofaciální fenotyp, včetně orofaciálních rozštěpů. TMEM107 protein je součástí tranzitní zóny primárních cílií a jeho působením dochází k regulaci transportu proteinů do cílie a následně zpět do buňky. *Tmem107*-deficientní jedinci vykazují projevy typické i pro jiné ciliopatie jako polydaktylii, vady ledvin, situs inversus a rozštěpy rtu a patra.

Závěrem lze shrnout, že dizertační práce přináší unikátní informace o kraniofaciálním vývoji chameleona jemenského a představuje tento druh jako nový potenciální model pro další evolučně-vývojově (EVO-DEVO) zaměřené studie. Dále pak naše výsledky charakterizují myší modely deficientní ve dvou klíčových genech pro kraniofaciální vývoj - *Cdk13* a *Tmem107*. Tyto poznatky přispějí k porozumění molekulární podstaty vrozených vývojových poruch vzniklých jejich mutacemi a mohou tak vést k prevenci jejich vzniku u lidí.

## Abstract

Development of the face is a sophisticated process regulated by myriad signalling pathways. Each step of facial development can be affected by various factors, resulting in facial malformations. The most frequent craniofacial defects in humans are cleft lip and cleft palate. Determination of new factors that contribute to their development would help clinicians prevent their initiation in a faster and more accurate way.

The first part of this thesis focuses on the morphogenesis of the secondary palate in the veiled chameleon, which forms large palatal shelves in contrast to other squamate reptiles; only a small physiological cleft remains in most adult animals. In the veiled chameleon, insufficient growth of palatal shelves towards the midline is caused by decreasing proliferation and overall changes in head dimensions during development. Decreased proliferation was associated with reduced synthesis of Sonic hedgehog (SHH) in the palatal shelves, and SHH probably also plays a role in their medio-lateral growth. Moreover, embryonic development of the chameleon palate was accompanied with the alteration in *Msx1*, *Meox2* and *Pax9* gene expression, which play important roles in mammalian palatogenesis.

The second part of the thesis focuses on mouse models with extensive craniofacial developmental defects caused by deficiency in two candidate genes. First, we analyzed a mouse model with a mutation in *Cdk13*. Mutation of this gene in humans triggers numerous developmental defects, including craniofacial abnormalities. This gene encodes the protein CDK13, a kinase that regulates transcription and alternative splicing. In a mouse model, a hypomorphic allele caused a mild craniofacial phenotype including cleft palate, while complete knockout resulted in midfacial structure deficiency along with cleft of the secondary palate.

A mouse with a mutation in *Tmem107* represents a model of three human syndromes with a typical craniofacial phenotype that includes orofacial clefts. This gene encodes a protein that is located in the transition zone of the primary cilium; it regulates protein trafficking to and from this organelle. In mice, phenotypic manifestations are very similar to humans, with polydactyly, kidney defects, situs inversus and cleft lip and palate.

In conclusion, our results provide novel information about craniofacial development of the veiled chameleon and introduce this species as a new model for evolutionary developmental biology (EVO-DEVO) studies. Moreover, our findings characterize two mouse models of human syndromes that could help to resolve molecular background of diseases caused by mutations in *CDK13* and *TMEM107* genes in humans.

## Aims

The main aim of this PhD thesis is to uncover new aspects of the complex molecular regulation during craniofacial development with focus on morphogenesis of orofacial structures including upper lip formation, secondary palate enclosure and regulation of odontogenesis.

The specific aims of the thesis are as follows:

- to reveal morphological and molecular characteristics during development of the secondary palate in the veiled chameleon;
- to determine in detail the phenotype of *Cdk13* mutant mice, with a focus on the evaluation of possible usage of this transgenic organism as a model to study developmental defects in congenital heart defects, dysmorphic facial features and intellectual developmental disorder (CHDFIDD) in humans;
- to specify the role of the TMEM107 in craniofacial development;
- to summarize the role of the primary cilium in development of the tooth.

Results

List of publications related to the topic of the thesis and author contributions:

1. HAMPL M, DUMKOVA J, KAVKOVA M, DOSEDELOVA H, BRYJOVA A, ZAHRADNICEK O, PYSZKO M, MACHOLAN M, ZIKMUND T, KAISER J and BUCHTOVA M. Polarized Sonic Hedgehog Protein Localization and a Shift in the Expression of Region-Specific Molecules Is Associated With the Secondary Palate Development in the Veiled Chameleon. *Frontiers in Cell and Developmental Biology*. 8:572. 2020.
   - writing the first draft of the manuscript and its revised forms
   - collection of embryos, dissection and processing of tissues
   - skeleton and cartilage staining
   - WISH probe synthesis
   - detection of gene and protein expression (IHC, WISH, qPCR, RNAScope)
   - taking pictures, their processing and figures preparation

2. NOVAKOVA M, HAMPL M, VRABEL D, PROCHAZKA J, PETREZSELYOVA S, PROCHAZKOVA M, SEDLACEK R, KAVKOVA M, ZIKMUND T, KAISER J, JUAN HC, Ming, FANN MJ, BUCHTOVA M, KOHOUTEK J. Mouse Model of Congenital Heart Defects, Dysmorphic Facial Features and Intellectual Developmental Disorders as a Result of Non-functional CDK13. *Frontiers in Cell and Developmental Biology*. 7:155. 2019.
   - writing draft of result parts of the manuscript
   - phenotype analysis
   - collection of embryos, dissection and processing of tissues
   - morphological analyses
   - IHC (Ki67, myosin, actin)
   - statistical analyses

- taking pictures, their processing and figures preparation

3. CELA P, HAMPL M, SHYLO NA, CHRISTOPHER KJ, KAVKOVA M, LANDOVA M, ZIKMUND T, WEATHERBEE SD, KAISER J, and BUCHTOVA M. Ciliopathy Protein Tmem107 Plays Multiple Roles in Craniofacial Development. *Journal of Dental Research*. 97 (1). 108-117. 2018.
   - writing draft of result parts and discussion section of the manuscript
   - phenotype analysis
   - IHC (PCNA, alpha tubulin, IFT88)
   - taking pictures, their processing and figures preparation

4. HAMPL M, CELA P, SZABO-ROGERS HL, KUNOVA BOSAKOVA M, DOSEDELOVA H, KREJCI P, BUCHTOVA M. Role of Primary Cilia in Odontogenesis. *Journal of Dental Research*. 96 (9). 965-974. 2017.
   - drafting the first version of manuscript and revised version
   - histological analyses
   - IHC (alpha tubulin, ARL13b)
   - taking pictures, their processing and figures preparation

1. **Polarized Sonic Hedgehog Protein Localization and a Shift in the Expression of Region-Specific Molecules Is Associated with the Secondary Palate Development in the Veiled Chameleon.**
   *Frontiers in Cell and Developmental Biology*. 8:572. 2020.

Morphology of the secondary palate differs among species based on the environment in which they live, how individuals communicate with each other and how they obtain and process the food. In this study, we described in detail morphology and development of the secondary palate during pre- and post-hatching phases of the veiled chameleon.

Early facial development is similar to other vertebrates including mammals. Palatal shelves develop from the maxillary prominence as their medial protrusions. During further development, they protrude directly horizontally toward each other unlike in mammals, where the palatal shelves grow first vertically down along the tongue and then reorient and grow further horizontally to finally fuse together. In chameleons, the opposite palatal shelves, in some animals, reach each other in the midline, but they do not fully fuse leaving the physiological cleft, as can be observed in birds.

The major secondary palate-forming bones in the veiled chameleon are palatines and pterygoids. These bones are also the first to ossify during development of chameleon`s head. The maxillary bones, which are the major palate forming bones in mammals and crocodilians, are reduced to more lateral position.

Localized proliferation in the dorsal maxillary prominence is likely not only a cellular process that contributes to directional growth of the palatal shelves. The polarized cellular localization of primary cilia and SHH protein was associated with the horizontal growth of the palatal shelves especially in the older pre-hatching developmental stages.

Moreover, the development of the secondary palate was coupled with the shift in *Meox2*, *Msx1* and *Pax9* gene expression along the rostro-caudal axis. Altogether, these findings could contribute to set up the veiled chameleon as a new model for craniofacial development, especially with focus on the EVO-DEVO aspects of the developmental biology.

2. **Mouse Model of Congenital Heart Defects, Dysmorphic Facial Features and Intellectual Developmental Disorders as a Result of Non-functional CDK13.**

*Frontiers in Cell and Developmental Biology*. 7:155. 2019.

Cyclin-dependent kinases (CDK) are enzymes, which are responsible for phosphorylation of other proteins and their function is dependent on binding to their partners called cyclins. They are generally separated into two groups: cell cycle-related kinases, which are control proteins of the cell cycle; and transcriptional cyclin-dependent kinases. CDK13 belongs to the group of transcriptional kinases and it is activated by binding to cyclin K. It phosphorylates RNA Polymerase II and thus affects binding diversity of the nuclear factors.

Recently, several studies uncovered the role of CDK13 mutations as inducing factor to cause developmental defects in human. Patients with a *CDK13* mutation are delayed in development and have intellectual disorder, heart and kidney defects and also craniofacial deformities. This phenotype is collectively known as Congenital Heart Defects, Dysmorphic Facial Features and Intellectual Developmental Disorder (CHDFIDD).

To model defects described in humans with *CDK13* mutations, two mouse models were developed; they have a similar phenotype to human patients, including delayed development, heart defects, and

developmental defects of kidney, liver and lungs. A hypomorphic *Cdk13* allele exhibited a milder craniofacial phenotype with cleft palate compared with the full *Cdk13* knockout. Hypomorph homozygous mutants produce non-functional CDK13 protein with only the N-terminus. In complete knockout, exons 3 and 4 were deleted and the allele is non-functional. The phenotype of the *Cdk13* knockout mouse was much stronger with extensive midfacial cleft with incomplete development of the upper lip and cleft palate. Although mutations in human are not lethal, hypomorph mutation was embryonically lethal at embryonic day 16.5 (E16.5), and complete knockout of the *CDK13* gene causes embryonic lethality at E14.5.

As the hypomorph CDK13 mutant mice share many phenotypical features with human patients, it seems to be promising model for future research focused on developmental defects caused by CDK13 mutations.

3. **Ciliopathy Protein Tmem107 Plays Multiple Roles in Craniofacial Development.**

*Journal of Dental Research*. 97 (1). 108-117. 2018.

Primary cilium is a solitary organelle situated on the cellular surface, which forms from the mother centriole. It consists of an axoneme covered by the cellular membrane and the basal body. These microtubule-based organelles can react to the mechanical and chemical stimuli but they also operate as a location where signalling pathways associate to each other. Primary cilia include receptors of main signaling pathways and transduce intracellular cascades to regulate differentiation, migration, and cell growth during development, but also in adulthood during disease induction and progression.

Primary cilium is separated from the cell by the transition zone that form semipermeable barrier, which regulates the protein content

of the primary cilium. One of the proteins enriched in this zone, is Transmembrane protein 107 (TMEM107). Mutation of the TMEM107 in human causes spectrum of diseases. The best described are Oral-facial-digital syndrome, Meckel-Gruber syndrome, or Joubert syndrome. These syndromes share typical features of ciliopathies: polydactyly, polycystic kidneys, situs inversus, and craniofacial defects including orofacial clefts. To discover, how TMEM107 mutation affects development, mouse model with mutated *Tmem107* has been developed. This mouse line share most of the developmental defects with human patients. *Tmem107*$^{-/-}$ mice were affected by a broad spectrum of craniofacial defects, including a shorter snout, expansion of the facial midline, cleft lip, extensive exencephaly and microphthalmia or anophthalmia. External abnormalities were accompanied by defects in skeletal structures, including ossification delay in several membranous bones, namely frontal and parietal. In some cases, tooth germs of incisors were either rudimentary or they were lost. Conversely, there were no apparent molar defects. TMEM107 mutant mice were prenatally lethal.

Alteration in the growth of palatal shelves resulted in clefting of the secondary palate. Palatal defects were caused by increased mesenchymal proliferation leading to early overgrowth of the palatal shelves, followed by defects in their horizontalization. Moreover, the expression of epithelial stemness marker SOX2 was altered in the palatal shelves of *Tmem107*$^{-/-}$ mice. The differences in mesenchymal SOX9 expression demonstrated enhanced neural crest cell migration.

Detailed analysis of primary cilia revealed region-specific changes in ciliary morphology accompanied by altered acetylated tubulin and IFT88 expression. Moreover, *Shh* and *Gli1* expression was increased in *Tmem107*$^{-/-}$ mice, as shown by ISH. On the other hand, *Shh* and *Gli1* expression in mutant embryos was decreased in the region of the developing incisors.

In conclusion, mutation of *Tmem107* causes very similar craniofacial phenotype to other ciliary proteins with the strongest effects observed in the anterior oral structures. In addition, *in vitro* functional tests revealed that primary cilia in cells isolated from the anterior palatal shelves presented the greatest response to ectopic morphogens. These results, together with the *in vivo* phenotype of transgenic mice, suggest that changes in primary cilium function cause region-specific alterations in crucial signalling pathways such as FGF and WNT.

## 4. Role of Primary Cilia in Odontogenesis.

*Journal of Dental Research*. 96 (9). 965-974. 2017.

Primary cilia have been associated with numerous physiological and pathological processes in human and also in other animals. As they function as a signaling hub to concentrate large number of different signaling molecules from various signaling pathways, their defective function leads often to diverse malformations and diseases. These cellular organelles are recently in the center of interest to study as they were associated with large number of scientific questions from different fields. Our aim was to review all recent information about primary cilia localization and function during odontogenesis and associated developmental defects.

As the primary cilium coordinates several signalling pathways essential for odontogenesis, ciliary defects can cause morphological abnormalities in tooth formation. Dental phenotypes, including supernumerary or missing teeth, enamel and dentin hypoplasia or crowding of teeth, caused by defective primary cilia are often part of ciliopathies. Both human patients and animal models with mutations in primary-cilium-specific genes have demonstrated that primary cilia play

a critical role in regulation of both the early odontogenesis and later differentiation of hard-tissue-producing cells.

**Conclusions**

This thesis aimed to explore cellular and molecular processes during palatogenesis using three different models of the developmental biology.

The main findings can be summarized in following points:

- The growth of the palatal shelves in chameleon continued during post-hatching stages and closure of the secondary palate can be observed in several adult animals.
- The massive proliferation of a multilayered oral epithelium and mesenchymal cells in the dorsal part of the palatal shelves underlined the initiation of their horizontal outgrowth and was decreased later in development.
- The polarized cellular localization of primary cilia and Sonic hedgehog protein was associated with horizontal growth of the palatal shelves.
- The development of large palatal shelves was coupled with the shift in *Meox2, Msx1*, and *Pax9* gene expression along the rostro-caudal axis.
- *Cdk13*-deficient animals exhibit overall developmental delay. altered development of multiple organs, partly resembling defects observed in patients with CHDFIDD syndrome
- Mutation in *Cdk13* caused incomplete secondary palate formation with variability in severity among *Cdk13*-deficient animals or complete absence of midfacial structures
- *Tmem107*$^{-/-}$ mice were affected by a broad spectrum of craniofacial defects, including shorter snout, expansion of the facial midline, cleft lip, extensive exencephaly, and microphthalmia or anophthalmia.
- External abnormalities in *Tmem107*$^{-/-}$ mice were accompanied by defects in skeletal structures, including ossification delay in

several membranous bones and enlargement of the nasal septum or defects in vomeronasal cartilage.

- Alteration in palatal shelves growth in *Tmem107*-deficient animals resulted in clefting of the secondary palate. Palatal defects were caused by increased mesenchymal proliferation leading to early overgrowth of palatal shelves followed by defects in their horizontalization.
- The expression of epithelial stemness marker SOX2 was altered in the palatal shelves of *Tmem107*$^{-/-}$ animals, and differences in mesenchymal SOX9 expression demonstrated the enhancement of neural crest migration.
- Analysis of primary cilia revealed region-specific changes in ciliary morphology accompanied by alteration of acetylated tubulin and IFT88 expression and *Shh* and *Gli1* expression was increased in *Tmem107*$^{-/-}$ animals.
- We summarized information about a role of ciliary genes during odontogenesis and discussed their morphological and functional abnormalities in the light of developmental defects in teeth.

# Curriculum Vitae

Name and surname: l

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**Education**

2015 – 2020

2013 – 2015

2010 – 2013

2006 – 2010

**Abroad studies and internships**

**Work experience**

**References:**

**Additional education**

**Other professional activities/Teaching**

**List of Publications**

**HAMPL M**, DUMKOVA J, KAVKOVA M, DOSEDELOVA H, BRYJOVA A, ZAHRADNICEK O, PYSZKO M, MACHOLAN M, ZIKMUND T, KAISER J and BUCHTOVA M. **Polarized Sonic Hedgehog Protein Localization and a Shift in the Expression of Region-Specific Molecules Is Associated With the Secondary Palate Development in the Veiled Chameleon.** *Frontiers in Cell and Developmental Biology*. 2020. IF: 5.206.

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**Conferences**

| | |
|---|---|
| July 2019 | poster, international congress ICVM 2019, Praha, Czech Republic |
| September 2018 | poster, international congress V4SDB Brno, 2018 |
| July 2018 | poster, international congress EMBO Workshop: Imaging Mouse Development, Heidelberg, Germany |
| October 2017 | poster, international congress BSDB Joint Meeting, Stockholm, Sweden |
| September 2017 | oral presentation, international congress Morphology 2017, Pilsen, Czech Republic |
| July 2016 | oral presentation, international congress ICVM 2016, Washington, D.C., USA |

**Grants/Fellowships**

| | |
|---|---|
| 2020 | Dean´s Award for the best PhD student |

| | |
|---|---|
| 2018 | International Mobility Program, IAPG CAS for transfer of biomedical innovations |
| 2017 | Award of Czech Anatomical Society |
| 2014 | Award of the Rector of the Masaryk University for Master´s students support, category C – MUNI/C/1004/2013 |

**Languages**

| | |
|---|---|
| English | active communication |
| Russian | passive communication |

**Other skills**

| | |
|---|---|
| Driving license | group B |
| PC | MS Office (Word, Excel, PowerPoint) |
| | Adobe Photoshop |
| | ImageJ |
| | LAS X (Leica Microscopes software), ZEN (Carl Zeiss Microscope Software) |