

THESIS FOR THE DEGREE OF DOCTOR OF *PHILOSOPHY* (PHD)

Investigations of new members of the signaling pathways during tooth development: the effects of Toll-like receptor 4 and Heat-shock protein 60.

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1.1 SCIENTIFIC BACKGROUND

Toll-like receptors are widely investigated in immunological aspects, but only a few articles are available about their roles during embryonic development under healthy conditions. Subsequently, to gain a new piece of the puzzle of embryogenesis and functions of TLRs, we focused on the functions of these receptors during tooth development, which area does not contain any available information about the function of Toll-like receptor 4. Concerning that TLR4 has a prominent function on mature odontoblasts, we aimed to investigate the expression pattern and possible function of it during the early stages of tooth development. On the other hand, Toll-like receptors have several endogenous and exogenous ligands which can activate the downstream signaling of NF- κ B and MAPK. Related to this, we investigated a possible endogenous ligand of it, which was the heat shock protein 60. According to earlier findings, it can activate the TLR signaling and/or it can modify the NF- κ B downstream signaling through the modification of IKK complex. A member of IKK complex has an individual role during the tooth development, which can attach directly to the heat shock protein 60. This connection leads to abnormal tooth development. Related to short summary, our goal was to detect the possible presence and role of TLR4 and Hsp 60 protein, which molecules probably can modify the tooth development.

1.2 ABBREVIATIONS

BBR: Boehringer blocking reagent

BCIP: 5-bromo-4-chloro-3-indolylphosphate

BMP: bone morphogenetic protein

BrdU: 5-bromo-2-deoxyuridine

BSA: bovine serum albumin

DAB: 3, 3'-diaminobenzidine

DMEM: Dulbecco's modified Eagle's medium

E*: embryonic day *

FGF: fibroblast growth factor

HMPG: high motility group protein

Hsp: heat shock protein

Hsp 25: heat shock protein 25

Hsp 60: heat shock protein 60

Hsp 86: heat shock protein 86

LPS: lipopolysaccharide

I κ B: inhibitor of κ B

IKK: inhibitor of κ B kinase

NBT: nitro blue tetrazolium

NF- κ B: nuclear factor- κ B

NMRI: mouse strain

MAPK: mitogen activated protein kinase

PBS: phosphate buffered saline

PFA: paraformaldehyde

PN: post-natal

SD: standard deviation

SHH: sonic hedgehog

SSC: saline-sodium-citrate buffer

TBS: Tris-buffered saline

TGF: transforming growth factor

TLR: toll-like receptor

TNF: tumor necrosis factor

TRIS: (hydroxymethyl)aminomethane
solution

WB: western blot

WNT: Wingless-related integration site
protein

2. REVIEW OF THE LITERATURE

2.1 Review of tooth development

Tooth development is carried out by reciprocal interactions between the epithelial cells of stomodeum and the neural crest derived ectomesenchymal tissue (Cobourne et al, 2010; Rothova et al, 2012). The reciprocal interactions are coordinated by continuous cross-talk between the neighbouring tissues of the tooth germ, similarly to the development of other ectodermal appendices (such as hair, nail). However, more than a dozen of signal transduction pathways take part in organogenesis, the following ubiquitous pathways play cardinal role during the tooth development: TGF, Notch, WNT, FGF, TNF and SHH pathways (Harada et al, 1999; Liu et al, 2008; Tummers et al, 2009; Felszeghy et al, 2010; Seidel et al, 2010). The literature divides the murine tooth development into three principal stages (Caton et al, 2009). These stages are: initial, morphological, and histodifferentiation parts.

- The initial part of the mouse incisor development starts at the embryonic day 11.5 (E11.5) (Harada et al, 2002). During the initial part, the underlying ectomesenchymal cells secrete FGF and BMP molecules, which causes the proliferation of cells of the oral epithelium at the territories of the dental lamina (Despina et al, 2009). Later the territory of the epithelial proliferation will be thicker and the daughter cells start to enter into the underlying mesenchyme. These migrating cells form the epithelial band, which is the first visible sign of tooth development (Maas et al, 1997).

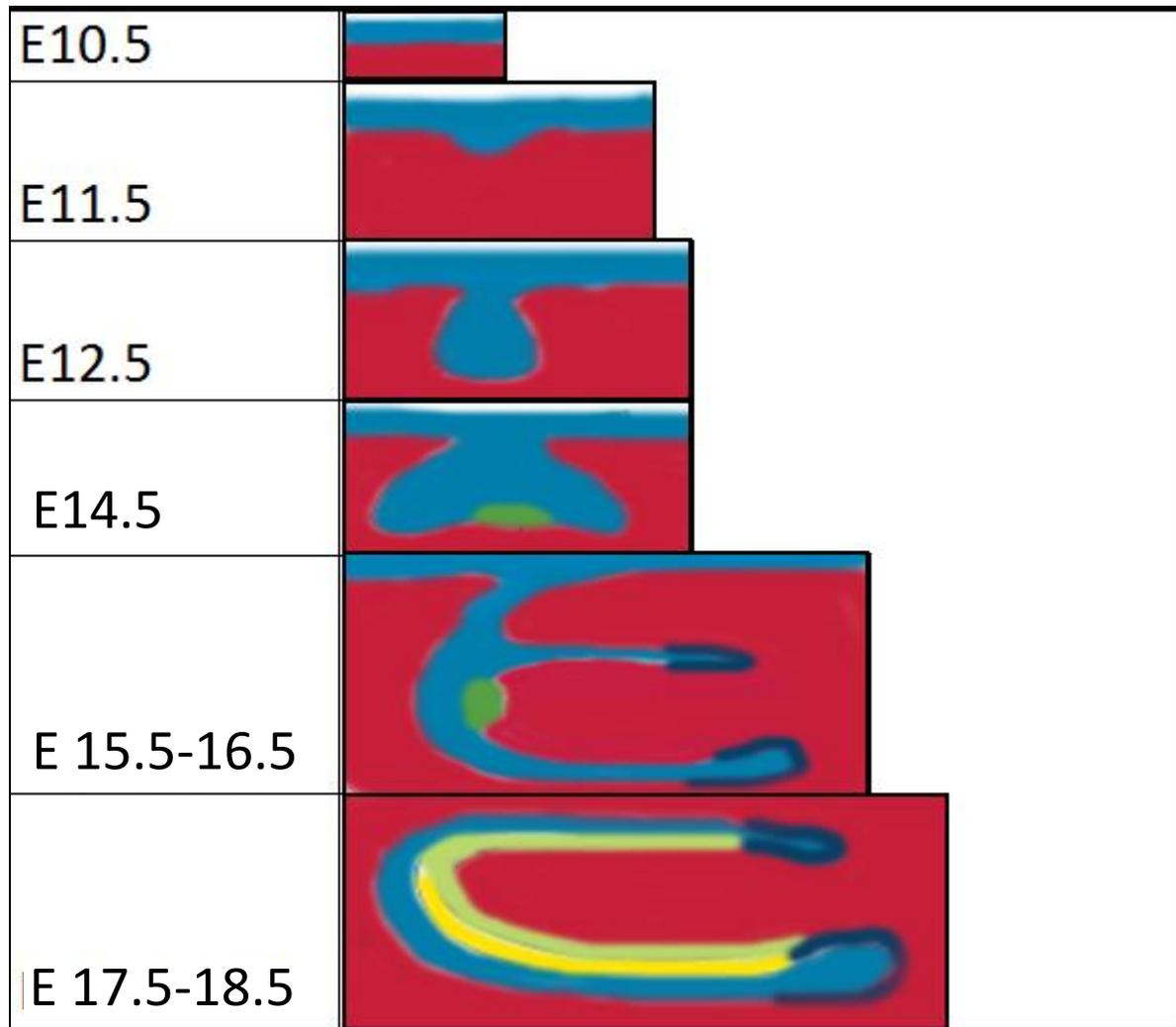
- The second phase of the tooth development is the morphogenesis (from E12.5 to E18.5). The literature divides the morphogenetic phase into bud, cap and bell stages (Jheon et al, 2013). Briefly, under the morphogenesis the cells of epithelial band form the enamel organ, which grows continuously into the underlying mesenchyme and form the shape of the teeth (Harada et al, 2002). The most proximal part of the enamel organ also forms the labial

and lingual roots of the tooth germs in murines, which parts contain pluripotent stem cell. These stem cells are responsible for the continuous growth of incisor teeth. Enamel organ encloses ectomesenchymal tissue and form the mesenchyme of the dental papilla (Despina et al, 2009). From the bell stage mesodermal structures accompany to the dental papilla and provide blood supply to dental papilla (Liu et al, 2014). The enamel organ is cover by mesenchymal cells, which form the dental follicle.

- During the histodifferentiation part of development the enamel organ and dental papilla form specialized cells, which form the hard tissues of the tooth (Caton et al, 2009). The morphological and histodifferentiation parts of the tooth development overlap with each other in time under the embryogenesis. During the histodifferentiation part, dental cell turn in highly specialized cell types, which can support only one function during the formation of the tooth germ. The enamel organ separates into several parts: inner and outer enamel epithelium, stratum intermedium, reticulum stellatum (Lesot et al, 2002). The ameloblasts originate from the inner enamel epithelium; they produce enamel matrix proteins only on the labial side of the tooth germs (Simmer et al, 2010). During the histodifferentiation of ameloblasts, the literature separates different stages of ameloblasts maturing. These stages are the followings: morphogenetic stage, histodifferentiation, presecretory, secretory, post secretory and protective stage. The secretory ameloblasts produce enamel matrix proteins, which is the first sign of enamel development, the ameloblastin is the specific marker of this cells. The inner enamel epithelium also forms the enamel knot. This is the key structure of the cuspal morphogenesis and act as a signaling centre (Jernwall et al, 2000; Simmer et al, 2010). The stratum intermedium take a part of the mineralization processes of the enamel matrix, the reticulum stellatum provides the nutrition of ameloblasts (Wöltgens et al, 1995). The outermost layer of dental papilla turns into odontoblasts, which cells are responsible for the

formation of dentin matrix (Mochida et al, 2009). After the birth of animals only the histodifferentiation stage is active during the lifetime of murine.

Table 1. Short summary of the tooth germ morphological development.



The morphogenesis of tooth development (**Table. 1**) starts with the dental placodes which is a thickening of oral epithelium (light blue territory) (E11.5). The condensation of ectomesenchymal cells (red territory) under the dental placod start to secrete chemoattractive molecules, which induce the invagination of the enamel epithelium (E12.5). This ectomesenchymal tissue forms the dental papilla embedded into the enamel organ during the cap stage (E14.5). The cervical and lingual loop is forming during the cap stage (indicated by

dark blue line). The epithelial pluripotent stem cell niches are present in these loops. The signaling center of the developing incisors is enamel knot (marked by dark green). The enamel knots coordinate the tooth crown morphogenesis. The light green line indicates the dentin forming odontoblasts, the yellow territory labels the enamel forming ameloblasts (from E16.5)

Figure 1. Histodifferentiation of enamel organ

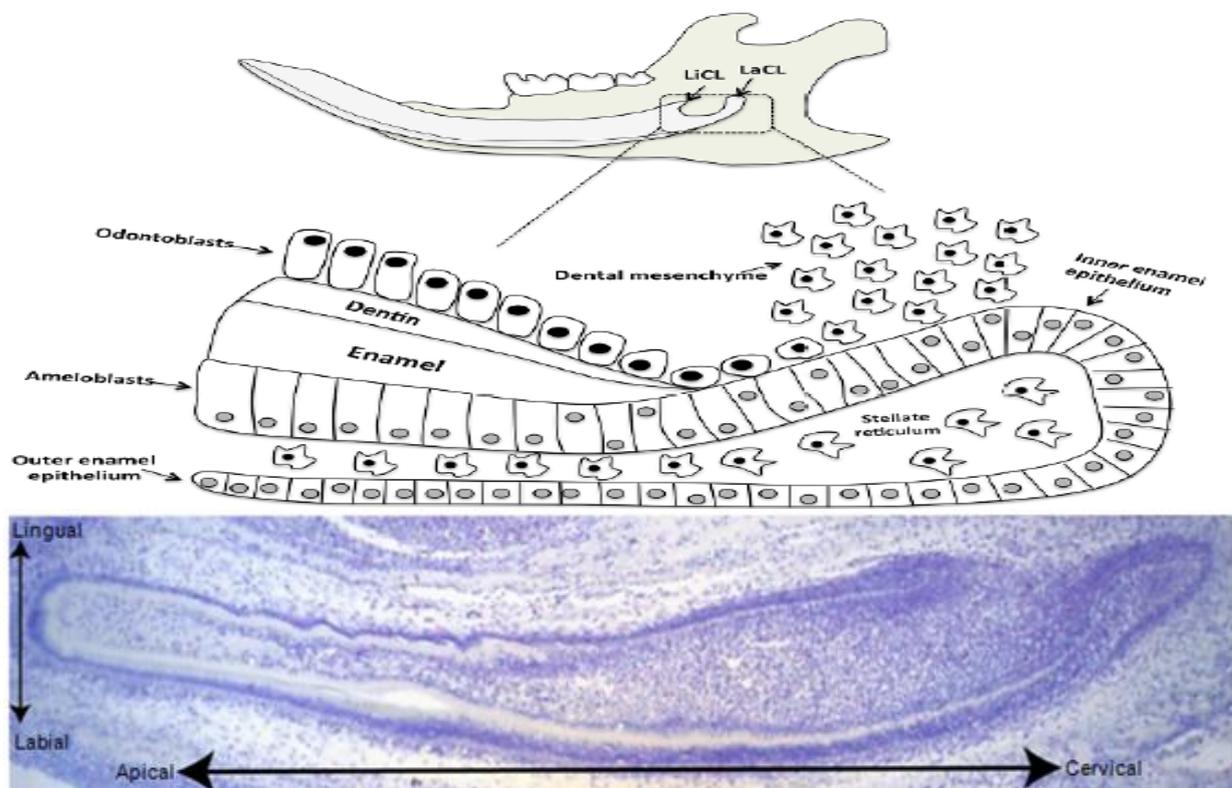


Figure 1. During histodifferentiation part the inner enamel organs separate into the outer enamel epithelium and inner enamel epithelium. Between these layer the reticulum stellatum is present, which contains pluripotent stem cells in the territories of cervical loops. The inner enamel epithelium differentiates into ameloblasts, which cells produce enamel matrix. The outset layer of dental mesenchyme turns into the odontoblastic layer, which produce dentin matrix. (Amer Sehic et al. 2015)

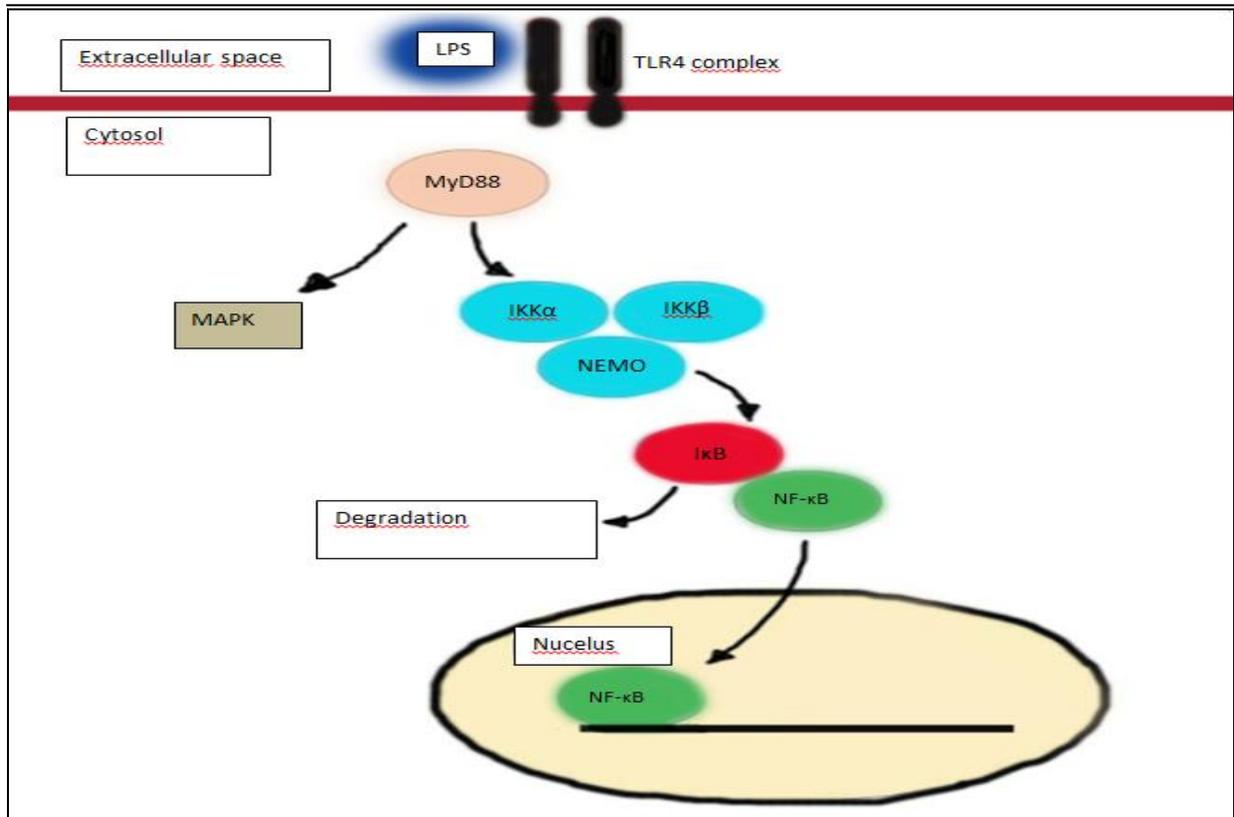
2.2 Roles of Toll-like receptors related to tooth

Toll-like receptors were identified almost 40 years ago related to the dorso-ventral axis development of *Drosophila*. Against this developmental aspect, nowadays the Toll-like receptors have a more prominent function in medical practice, namely the immunity. The specific molecular components of bacteria and viruses is screened by different TLRs. Related to this monitoring, the most prominent function of TLRs are the moderation of inflammatory processes. Each TLRs have a specific ligand, however generally they can recognize a wide spectrum of agonists. According to the literature we found experimental data about the role of TLR4 signaling during the inflammation of dental pulp (He et al, 2012, Horst et al, 2009, He et al, 2013). An important function of TLR4 in our focus is the modification of the mineralization processes of hard tissues such as bone, dentin (Sheng et al, 1999). Subsequently, numerous Toll-like receptors (TLR2; 4; 7; 8; 9) are presents in odontoblasts, which can identify different antigens, since the odontoblasts are the first vital structures, which can identify pathogens under caries (Lu et al, 2008, Fargues et al, 2011, Rassa et al, 2003, Mutoh et al, 2007, Pääkkönen et al, 2014). The downstream signaling pathways of TLRs can influence the activity and the life cycle of odontoblasts through the MAPK pathway (Wang et al, 2015). This modification of odontoblasts activity can result the alteration of dentin matrix synthesis proteins (Farges et al, 2009). TLR signaling also may activate the NF- κ B signaling pathway, through the degradation of I κ B. In absence of I κ B, the NF- κ B dimer can activate transcription of several genes, which cause the immunresponse against the pathogen.

The widely accepted specific ligand of the TLR4 is the lipopolysaccharide (LPS), a component of the bacterial cell wall (Lu et al, 2008; Liu et al, 2014). The activation of TLR4 complex (MD-2, CD14 and TLR4) causes the degradation of the I κ B molecule, which causes activation of the downstream signaling pathway of NF- κ B and MAPK (Lu et al, 2008, Hayden et al, 2008). Nevertheless, not only particles of pathogen agents (as exogenous

ligand), but also different endogenous ligands, for example heat shock proteins (Hsp), Gp96; proteoglycans can activate the TLRs. One widely accepted group of a non-specific activator of the TLRs can be different heat shock proteins (Lehnardt et al, 2008, Okun et al, 2011, Ohashi et al, 2000).

Figure 2



The LPS a bacterial component can activate the Toll-like receptor 4 as an exogenous ligand (Fig 2.). The activation causes the phosphorylation (black arrows) of IKK complex (light blue complex) which cause the degradation of IκB. In the absence of IκB the NF-κB (green territory) can enter into the nucleus and activates the transcription of early response elements (INF, interleukins). The MAPK can be also the effector of TLR4 signalling, modifying the life cycle of cells.

2.3 Role and presence of heat shock proteins during tooth development

The members of Hsp family are ubiquitous proteins in every cell, since the Hsp-s act as housekeeping proteins, and the absence of Hsps cause lethal developmental failures. (Richter et al, 2010). We can make difference between these proteins according to the molecular weights and cellular localization (Beere et al, 2005; Zhu et al, 2010). Numerous Hsps have been reported related to embryogenesis in recent years, however originally the literature describes it as stress inducible proteins (Tutar et al, 2010). The Hsp expression seems to be cardinal in of the balance of proliferation and apoptosis under embryogenesis (Zhu et al, 2010).

During the tooth development several heat shock proteins were investigated. The expression pattern and possible functions of heat shock proteins 25, 27, 86, Hsc73 and Hsj2 were already described (Lee et al, 2009; Ohshima et al. 2000; Nakasone N et al. 2006; Wada et al. 2002; Leonardi et al. 2004). The Hsp 25 has been studied the most in mice incisors which can increase the proliferation rate of dental cells and present basically in the enamel organ (Lee et al, 2009; Ohshima et al. 2000). The odontoblasts showed strong Hsp 25 expression, the cells of dental pulp, preodontoblasts and ameloblasts were transiently positive. The Hsp 27 was detected in the early stage of the tooth development which can modify the morphological development of the tooth (Leonardi et al. 2004). Screening online databases the mRNA expression pattern of Hsp 60 changes dramatically, which indicates the possible role it during tooth development (Allen Institute for Brain Science, Allen Developing Mouse Brain Atlas, <http://developingmouse.brain-map.org>).

The Hsp 60, also a member of heat shock proteins family, a highly conserved molecule, which is expressed in prokaryotic and eukaryotic cells (Chun et al, 2010). The majority of Hsp 60 is localized in the mitochondria, approximately 20% of the total amount is

present in the cytoplasm and under healthy condition only a few percentages is secreted into the extracellular space (Calderwood et al, 2007). Hsp 60 has several and diverse functions related to the localization. The mitochondrial Hsp 60 has ability to help in the refolding of proteins, which are translocating into the mitochondria.

The cytosolic Hsp 60 is able help the folding of the proteins and it can play role in the modification of the NF- κ B signal pathway (Li et al, 2005) through IKK. The IKK has 3 parts: IKK α , IKK β and IKK γ (Zandi et al, 1997). According to the literature the free cytosolic Hsp 60 can attach to IKK α , which has an NF- κ B independent role during the early stage of embryonic development (Ohazama et al, 2004). The IKK α influencing the invagination of the ectodermal derived structures (tooth germ, whiskers) into the underlying mesenchyme, and absence of it can cause abnormal tooth phenotype (Ohazama et al, 2004; Sil et al, 2004). Hypoxia, fever increases the amount of Hsp within minutes and the intracellular Hsp can act as pre- and pro-apoptotic protein. The high concentration of extracellular Hsp 60 acts as a danger signal during stress (Morimoto et al, 1993).

Focused on the pathological environment of fetus, the relative high amount of Hsp 60 possibly can activate the TLR4 on the tooth germ, which may affect the morphological development or mineralization of tooth germs (Lehnardt et al, 2008).

3. AIMS OF THE STUDIES

- Our first aim was to investigate the putative presence and role of TLR4 during the tooth development.
- Our second goal was to detect any possible endogenous ligand of TLR4 which can activate it during embryonic development.

4. MATERIALS AND METHODS

4.1 Animal care

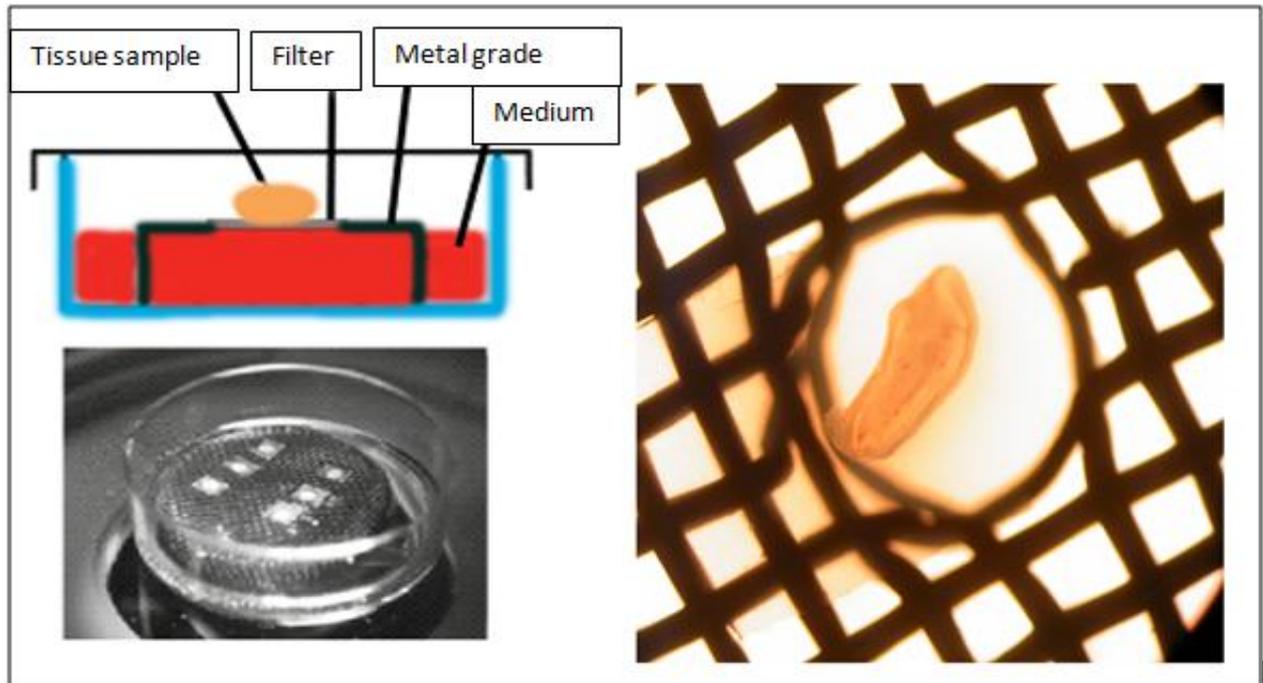
All experimental procedures followed the guidelines of the Animal Care and Use Committee of the University of Debrecen [DE FSZ/2010/10]. Pregnant females were euthanized according to these guidelines. Pregnant mice were killed by cervical dislocation and embryos were sacrificed by decapitation. Embryonic age was estimated using the appearance of the vaginal plug (E0.5), and from their exterior features.

4.2 Organoid *in vitro* culturing

According to earlier articles, we performed Trowel-type tissue culture taken from from 16.5-day old lower mice incisors (Harada et al, 1999). During isolation we prepared two of the incisors under Nikon SMZ 1000 stereomicroscope (Nikon Corporation, Tokyo, Japan) in 15 minutes long time period. Special attention has been paid to remove all the extra tissue surrounding the incisor tooth germs to avoid skewed data in our further analyses. The preparation of materials and dissection of tissues was carried out under sterile condition and was performed as previously described. Only the unwounded and healthy tooth germs were selected for culturing. Tissues were placed on 0.1- μ m pore-size nucleopore filters (Sigma, St. Louis, MO, USA) supported by metal grids in a humidified atmosphere of 5% CO₂ in air at 37 °C. One of the incisors was used as the treated explant and the pair of it was control. Both treated and control were excised from the same mouse, ensuring that the alteration of the development stage of these tooth germs were minimal. The culture medium consisted of 15% fetal bovine serum (Gibco Brl, Gaithersburg, MD, USA) in DMEM (Gibco Brl, Gaithersburg, MD USA). 1 μ g/mL LPS (InvivoGen, San Diego, USA) or 1 μ g/mL Hsp 60 (Abcam, Cambridge, UK) was added to the medium. The culture medium was changed on the third day

of culturing (after 48 hours), and the experiment was terminated on the fifth day. The data of *in vitro* culturing was based at least three different experiments.

Figure 3. Trowel-type tissue culture



Tissue explant is placed on a membrane, which is situated on the opening of the metal grade. The explants are supplied by the medium, but the superior surface is in contact with air. This type of *in vitro* culturing is suitable for following the morphological development of the tooth germ. Another important feature of this culturing, is the possibility of add different proteins temporally into the medium for visualization of the possible effects of this modification.

4.3 Morphological analysis

In order to detect the exact alteration of Hsp 60 treated tooth germ, we performed morphological analysis. We investigated the angle closed by the labial, lingual loops, and the enamel knot. We used *in vitro* data from the tooth germ, in order to avoid the shrinkage of the histological section related to the histochemical process.

4.4 ALP assay

Mineralization process of E16.5 day-old tooth germs were determined by alkaline phosphatase (ALP) activity assay and alizarin-red staining. Samples from the treated and control groups were stored at -70°C in ALP assay buffer. Samples were sonicated by pulsing burst for 30 sec at 40 A (Cole-Parmer, Illinois, USA). After centrifugation at 10.000g for 10 min at 4°C , supernatants with equal protein concentrations were used for enzyme activity measurements. ALP activity assay (Abcam, Cambridge, UK) was performed following the manufacturer's protocol

4.5 Immunohistochemistry, Western Blot and histochemistry

Western blot

The applied exogenous Hsp 60 protein (Abcam, Cambridge, UK) was tested by a monoclonal anti-Hsp 60 antibody (Thermo Scientific, Rockford, IL, USA) to qualify the specificity. WB experiments were carried out on isolated mouse tooth germs from E13.5 to E18.5 stages. The tooth germs remained intact, and the surface of the tooth germs did not contain connective tissue. Isolated tooth germs were placed in 50 μL homogenisation buffer containing 50 mM Tris-HCl buffer (pH 7.0), 10 $\mu\text{g}/\text{mL}$ Gordox, 10 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenylmethylsulphonyl-fluoride, 5 mM benzamidine, and 10 $\mu\text{g}/\text{mL}$ trypsin inhibitor. Starting the procedure tooth germs were sonicated by pulsing burst (Cole-Parmer, East Bunker Court Vernon Hills, IL, USA). For WB, total cell lysates were used. Samples for SDS-PAGE were prepared by the addition of two-fold concentrated electrophoresis sample buffer to cell lysates to equalise the protein concentration in samples, followed by boiling for 10 minutes. 10-20 μg of protein was separated by 7.5% SDS-PAGE gel for detection of Hsp 60 and actin. Proteins were electrophoretically transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in PBS for 1 hour at room temperature, membranes were washed and exposed to

the primary antibodies overnight at 4 °C. Monoclonal anti-Hsp 60 antibody (Thermo Scientific, Rockford, IL, USA), monoclonal anti-TLR4 antibody (Abcam, Cambridge, UK) and monoclonal anti-actin antibody (Sigma, St. Louis, MO, USA) in 1:10.000 were used. After washing for 3x10 minutes in PBST, membranes were incubated with anti-mouse IgG secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA) in 1:1500 dilution for 1 hour at room temperature. Signals were detected by enhanced chemiluminescence (Millipore, Temecula, CA, USA) according to the manufacturer's instructions. Signals were manually developed on X-ray films.

Immunohistochemical detection of TLR4.

Tooth germs were fixed in Sainte-Marie fixative for 30 min at 4 °C immediately after termination of *in vitro* culturing. Samples were washed for 10 minutes in PBS 3 times. Whole mount tooth germs were incubated by Vectastain Elite ABC Kit (Vector Laboratories Ltd., Peterborough, UK) to label the attached LPS-EB biotin. Control samples were stained in the same way, but Vectastain Elite ABC Kit was replaced with PBS. During the visualization of the immunoreaction we used DAB (Vector Laboratories Ltd., Peterborough, UK) for conventional light microscopy. The control sections originate from the same incisor in each case, these sections were immunostained in the same way, but the primary antibody was omitted and replaced with PBS. No signal was recorded from control sections. DAB immunoprecipitations were evaluated independently by 3 researchers on immunostained samples.

Immunohistochemical detection of Hsp 60.

Samples from E10.5 to E18.5 were fixed in Sainte Marie's solution, the earlier stages of tooth development (from E16.5) we demineralised samples in 10% EDTA. During dehydration graded series of alcohol and xylol was used. Samples were embedded into

paraffin. Section of 5 μm were made, followed deparaffinization and we preincubated in 1% normal horse serum in PBS for 30 min at 24 °C to prevent non-specific binding of the primary antibody. Previously to block the endogen peroxidase activity samples were treated with 3% H_2O_2 for 5 minutes. We immunostained our samples with anti-Hsp 60 antibody at 1:200 in PBS (Thermo Scientific, Rockford, IL, USA) overnight, at 4 °C, as secondary antibody anti-mouse Ig-G (Vector Laboratories Ltd., Peterborough, UK) at 1:400 in PBS was applied for 2 hours at room temperature, and visualised by Vectastain Elite ABC Kit (Vector Laboratories Ltd., Peterborough, UK) according to the manufacturer's protocol. The control sections originate from the same incisor in each case, these sections were immunostained in the same way, but the primary antibody was omitted and replaced with PBS. No signal was recorded from control sections. Immunoreaction were visualised with DAB.

The histological sections of tooth germs from *in vitro* culturing were stained with picosirius F3B as described in the literature (Constantine et al, 1968).

4.6 *In situ* hybridization

During the *in situ* hybridization pregnant NMRI animals were anaesthetised with sodium-pentobarbital (applied dose 50 mg/kg), embryos were dissected in PBS on ice. Probes were designed and used according to the manufacturer's protocol (Roche, Mannheim, Germany) as described earlier (Felszeghy et al, 2010; Girard et al, 2011; Zimmermann et al, 2013). PCR primer's sequences were chosen for regions containing exons from 7 to 12 and the 3'UTR regions using the mouse Hsp 60 mRNA (HSPD1, GenBank accession No: NM_010477.4) and ameloblastin mRNA (AMBN, GenBank accession No: NM_016519.5) as a template. The 'sense' primer was flanked by the T3 sequence, and the 'antisense' primer contained the T7 primer sequence at the 5' ends. The sequences of primers were:

Hsp 60 (GenBank accession No: NM_010477.4)

- T3 flanked sense: 5'-ATTAACCCTCACTAAAGGTCCCTGCTCTTGAAATTGCT

- T7 flanked antisense: 5'- AATACGACTCACTATAGGCTCCACAGAAAGGCTGCTTC

Ameloblastin (GenBank accession No: NM_016519.5)

- T3 flanked sense: 5'-AATTAACCCTCACTAAAGGCAGAAGGCTCTCCACT GCAA

- T7 flanked antisense: 5'-TAATACGACTCACTATAGGAGCAGTCAGGGTTTTCCACC

Probes were manufactured by Integrated DNA Technologies Inc., (Coralville, IO, USA). Dissected 16.5 day-old tooth germs were fixed in RNA-ase free 4 % paraformaldehyde in PBS for overnight at 4°C. After fixation, samples were washed for 10 minutes in PBS 3 times, incubated for 15 minutes in 0.1 m TPBS, finally we washed in PBS. Proteinase K (4 µg/mL) was used for digestion in TE buffer for 20 minutes at 37 °C. Samples were prehybridized for 30 minutes at 60 °C, then hybridized with the respective probes for over night at 70 °C. The hybridization buffer contained 50 % formamide, 5× SSC, 10 % Dextran sulfate, Denhardt's solution and 2.5 mg/mL probes. After incubation, sections were washed in 2× SSC, SSC and 0.2× SSC, 20 min each. Then we used 20 µg RNase A in 1 mL NTE for 30 min in 37 °C to break down the unhybridized probes. Finally samples were washed 3 times in TBS containing 0.1% Triton-X 100. During the detection blocking reagent contained 1 % Boehringer Blocking Reagent, 10 % goat serum in TBS. Alkaline-phosphatase-conjugated anti-digoxigenin (Roche, Mannheim, Germany) was applied for 8 h at 4 °C. Samples were washed in TBS 3 times for 10-10 minutes. Detection was performed with 20 µL NBT/BCIP (Roche, Mannheim, Germany) in 1 mL TRIS. After the colour development samples were washed in TRIS and stored in Scale A2 solution till documentation.

4.7 RT-qPCR

RT-qPCR was performed as described earlier (Szántó et al, 2014) with minor modifications. Briefly, total RNA was prepared using Trizol reagent (Invitrogen, Waltham, USA) according to the manufacturer's instructions from 7-7 LPS treated and control samples. We removed the lingual and labial loops of tooth germs while the tissue of interest remained intact, which was immediately placed for RT-qPCR experiments. The samples formed 2 pools, which were used during the RT-qPCR. cDNA was synthesized from 200 ng RNA sample using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR reactions contained 10 ng cDNA from each samples. The quantitative PCR reactions were performed using the LightCycler 480 system (Roche, Basel, Switzerland) and a qPCR supermix (PCRBiosystems, London, UK) with the following primers: ameloblastin (fwd: 5'- CATGCAGGACTTCTTGCTTTC; rev: 5'-GGTGCACTTTGTTTCCAGGTA) and cyclophilin as control (fwd: 5'-TGGAGAGCACCAAGACAGACA; rev: 5'-TGCCGGAGTCGACAATGAT). Ameloblastin expression was normalized for cyclophilin expression. In all cases qPCR reactions were performed on untranscribed RNA to verify the absence of genomic DNA contamination.

4.8 BrdU Assay

At the case of the *in vitro* culturing of Hsp 60 treated samples 10 µL BrdU labelling reagent (Life Technologies, Carlsbad, CA, USA) was administrated into the culture medium for 2 hours prior to fixation of the samples (Felszeghy et al. 2010). The samples were fixed in Sainte-Marie's fixative at 4°C for 30 minutes then dehydrated in graded series of ethanol and embedded in paraffin. Sections were made in the sagittal plane at 5 µm and processed for further immunohistological analysis. The incorporated BrdU was immunodetected by secondary antibody according to DAB method. After the developing of the DAB reaction, sections were washed in PBS 3 times. The positive control (thymus) was insured by the BrdU

Detection Kit (Zymed, Carlsbad, CA, USA). Sections were counterstained with DMMK provided by the BrdU Detection Kit.

4.9 ELISA

Tooth germs from the organoid culture were put and stored in RIPA buffer at -70 °C immediately after the culturing. During the processing of samples tooth germs were mechanically homogenized in TRIS-glycine buffer containing 1% SDS and protease inhibitors. Protein concentration of the tooth germs was measured with BCA assay. NUNC Maxisorp plates (Nunc Intermed, Copenhagen, Denmark) were coated with 10 µg protein/well in coating buffer (15mM Na₂CO₃, 35 mM NaHCO₃, 0.02% NaN₃, pH=9,6). Free binding surfaces of the polystyrene well were blocked with 1% BSA, followed by anti-collagen X primary antibody (Abcam, Cambridge, UK). During the detection of the primary antibody we used horse radish peroxidase-conjugated goat-anti-rabbit IgG as secondary antibody (DAKO, Glostrup, Denmark). The color reaction was developed with o-phenylene-diamine substrate and absorbency was measured at 492 nm with a microplate reader (Felszeghy et al, 2000). The positive control of the reaction was determined by the same procedure, on epiphyseal cartilage.

4.10 Alizarin-red staining

The tooth explants were fixed in Saint-Marie's fixative for 2 hours at room temperature, and after washing in deionized water the tooth germs were stained with Alizarin-red (Sigma Aldrich, St. Louis MO, USA) for 10 min at room temperature (Fraser et al, 2013). Excess dye was removed and washed with 20 % glycerol for 5 minutes at room temperature. Samples were stored in Scale A2 solution for 10 minutes before documentation.

4.11 Data analysis and image capturing

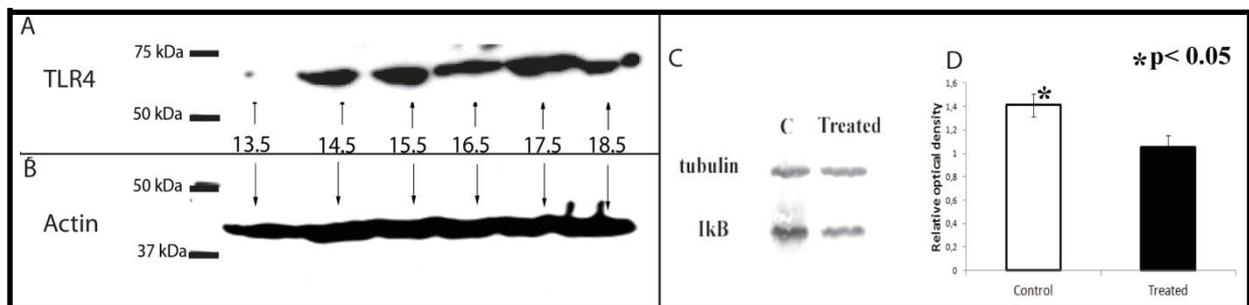
Images were captured convectional light microscopy (Nikon Eclipse E800, Nikon Corporation, Tokyo, Japan). The images acquired were representative of all the tissue sections examined. For documentation, images were processed by Adobe PhotoShop Software CS4 (Adobe Systems Inc., San Jose, CA, USA). Data of morphological analysis was measured with ImageJ 1.46 (National Institutes of Health, Maryland, USA). For statistical analysis, 7-7 control and treated samples from at least 3 independent experiments were compared with statistical analysis at the case of BrdU incorporation test. Data are expressed as mean \pm SEM. Statistical analysis was performed by Mann-Whitney test. For statistical analysis of ELISA and I κ B degradation (WB) at least 3 individual samples from 3 different culturing groups were used. Where applicable, data were expressed as mean \pm SEM. Statistical analysis was performed by Student's T test, where statistical method reported significant differences among the groups ($p < 0.05$).

5. RESULTS AND DISCUSSION

5.1 Functionally active TLR4 presented from the cap stage of tooth development

Protein expression of TLR4 was detected from the bell stages (from E14.5 to E18.5 stages) of the developing tooth (**Figure 4 A inset**). LPS addition in the applied dose to the culturing medium resulted significant reduction of the amount of I κ B in the case of the 16.5 day-old tooth germs (**Figure 4 C inset**). Our result was normalized to the amount of tubulin.

Figure 4. The developing tooth germs contain active TLR4 from the bell stage.



TLR4 expresses from the cap stage of tooth development. Toll-like receptor 4 presents continuously from E14.5 in the developing tooth germ **A inset**. **B inset** shows the loading control. LPS treatment decreases the amount of I κ B (**C, D insets**). Data represent the mean of optical density \pm SD (**D inset**); the difference is significant between the samples ($p < 0.05$).

5.2 LPS accumulation was detected in enamel organ and in preodontoblasts

The whole mount samples showed strong immunoreaction against biotin conjugated LPS in the enamel organ of the treated tooth germs. After histological processing of samples, the following layers contained LPS: presecretory, secretory ameloblasts, stratum intermedium and preodontoblasts (**Figure 5 A-B inset**). Interestingly, the postsecretory ameloblasts did not

contain LPS. Concerning, that the LPS is a specific ligand of TLR4, these structures contain active TLR4 in case of 16.5 day-old tooth germs.

Figure 5. Structures of enamel organ and preodontoblasts took up biotin labelled LPS.

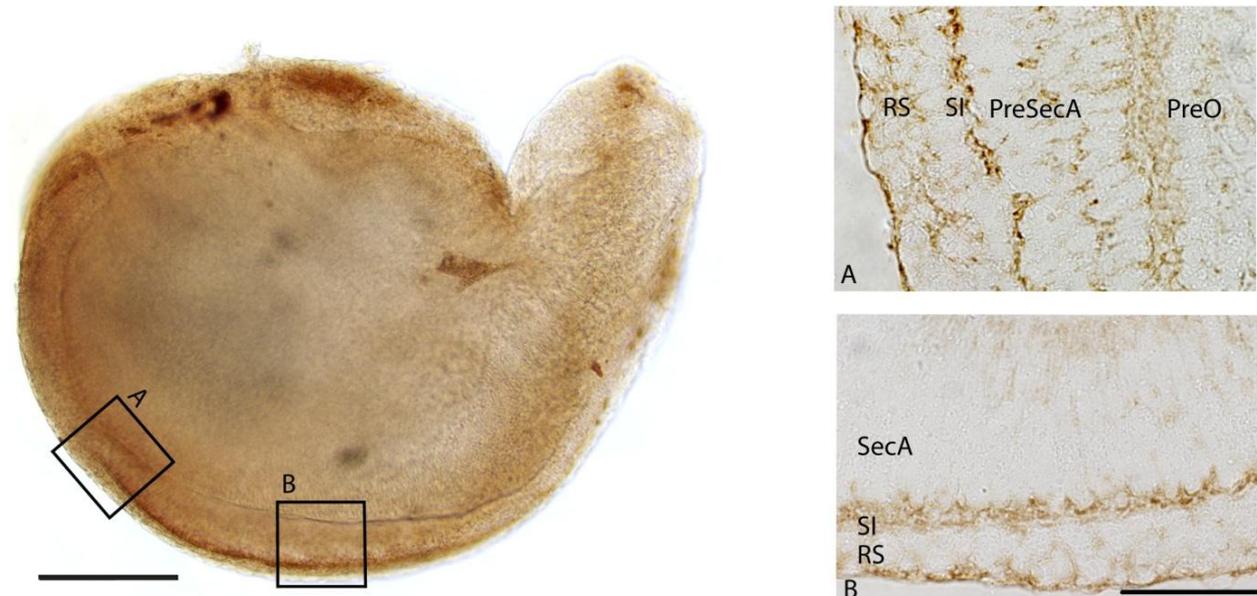


Figure 5. Structures of enamel organ and preodontoblasts took up biotin labelled LPS.

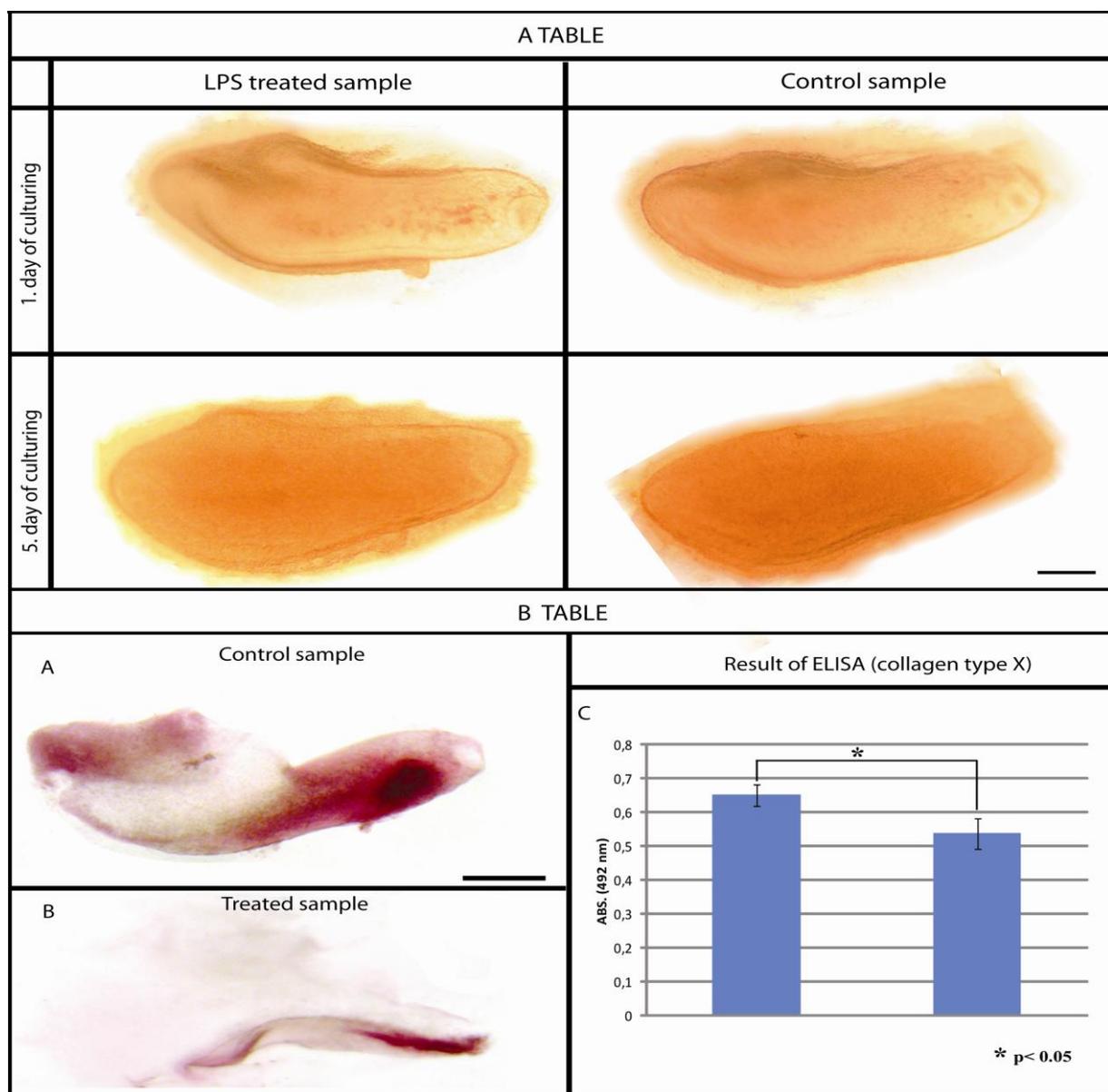
Immunohistochemistry shows strong immunoreactions in the labial part of the tooth germs against LPS (**Figure 5.**) Scale bar: 50 μm . The higher magnification (**A inset**) presents the DAB precipitation in the following structures: presecretory ameloblasts (PreSecA), preodontoblasts (PreO), stratum intermedium (SI) and reticulum stellatum (RS). **B inset** indicates the position of LPS in the basal part of secretory ameloblasts (SecA), stratum intermedium (SI) and reticulum stellatum (RS). Scale bar: 20 μm .

5.3 LPS inhibited the mineralization of hard tissues of tooth germ, without any morphological changes

No morphological alternation was detected in the tooth germs after LPS administration (**Fig 6, A table**). Histochemical staining was used to detect the mineralization process (**Fig 6, B table, A-B**). In control samples strong extracellular Ca^{2+} deposits were demonstrated with

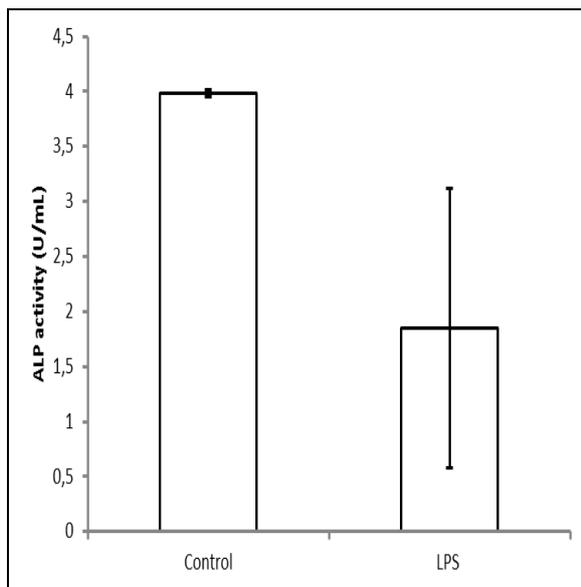
alizarin-red in the dentin and enamel. LPS treated tooth germs showed obviously weaker signal in the same structures. LPS treated tooth germ showed no obvious mineralization either lingual or labial side. ELISA was performed in order to investigate the amount of type X collagen. The amount of it decreased significantly related to LPS treatment. (**Figure 6 B table, C inset**), this result coincides with the result of alizarin-red staining and ALP assay (**Table 2**).

Figure 6. The LPS treatment did not alter the morphology, but inhibited the mineralization of hard tissues of the tooth germ.



We did not find any obvious morphological alteration between our samples during the *in vitro* culturing (**Fig 6. A table**). Scale bar: 100 μ m. The LPS treatment decreased the mineralization of hard tissues of the tooth germ (**B table**). The alizarin-red staining showed strong mineralization in the case of the control samples (**B table, A inset**), the treated samples contain Ca^{2+} only in the enamel organ (**B table, B inset**). Scale bar 100 μ m. The amount of type X collagen decreased in the treated tooth germs (**B table, C inset**). Data represents the mean of absorbance (ABS) \pm SD, ($p < 0.05$).

Table 2. shows the effect of LPS treating on the activation of ALP activity. Significantly decreased alkaline phosphatase activity was detected by ALP activity assay.



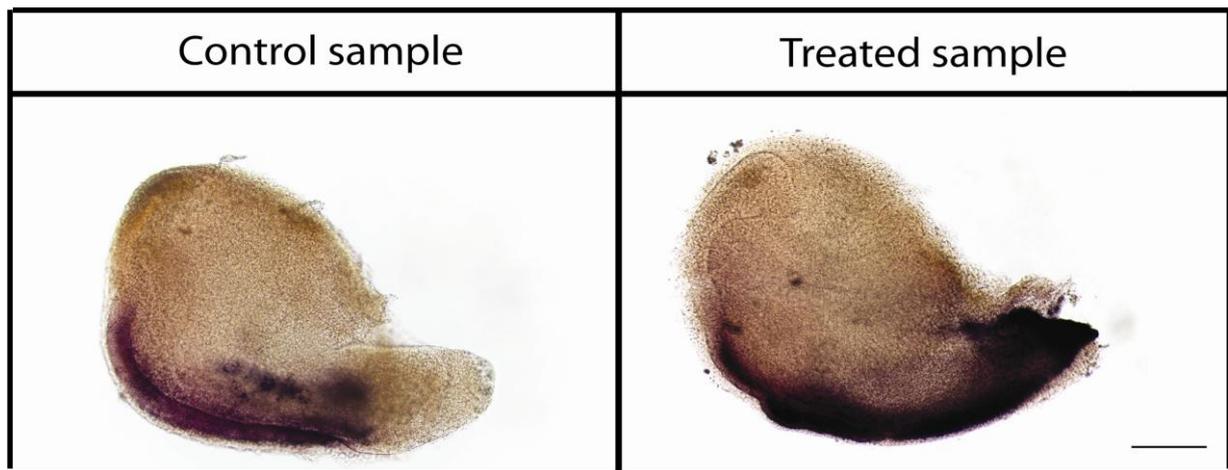
LPS treatment caused significant decreased activity of ALP during the culturing. According to the analysis of data from control and LPS treated samples the alkaline phosphates activity decreased dramatically, which also indicates the modification of mineralization of tooth germs.

5.4 LPS treatment can elevate the amount of ameloblastin mRNA

For investigation of possible effect of LPS on ameloblasts, we performed *in situ* hybridization against ameloblastin RNA. The ameloblastin is a specific marker of the secretory ameloblasts and indicates the maturation of these cells. Higher ameloblastin mRNA expression was

detected in the case of LPS treated samples. It is noteworthy that presence of ameloblastin was visible only in the enamel matrix with this technique both in control and LPS treated cultures (**Figure 7**). Data of **Table 3** (RT-qPCR) indicates the significantly enhanced synthesis of ameloblastin mRNA, which coincide the result of in situ hybridization.

Figure 7. The LPS alters the maturation of ameloblasts.



In situ hybridization showing levels of ameloblastin mRNA in the enamel matrix. LPS treatment increase obviously the amount of ameloblastin mRNA in the enamel organ. Scale bar: 50 μ m.

Table 3. LPS treatment significantly increased the amount of ameloblastin mRNA.

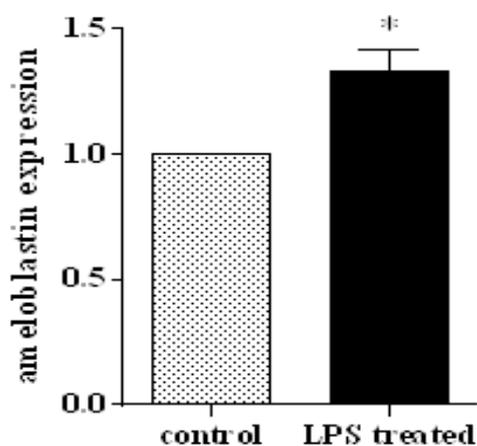


Table 3. shows the ameloblastin mRNA expression, determined by RT-qPCR, asterisks indicate significant differences (paired *t*-test; $p < 0.05$, (n: 7-7)) between LPS treated and control samples.

5.5 Hsp 60 is present in high levels in the structures of the enamel organ and in odontoblasts

We used Western blot and immunohistochemistry methods to detect Hsp 60 protein in tooth germs from E13.5 to E18.5. The results showed continuous and strong expression of Hsp 60 from the cap stage of enamel organ development (**Figure 8**). The first appearance of Hsp 60 was detected by only a weak DAB signal in the epithelial band (EB) during the initial stage of the tooth development (E11.5) (**Figures 8 B**). During the bud and cap stage (E13.5-E15.5; **Figures 8 C-F**), the inner enamel epithelium (IEE) and outer enamel epithelium (OEE) and enamel knot (EK) of the enamel organ showed strong immunoreaction against Hsp 60. The dental papilla (DP) and dental follicle (DF) contain weak signal (**Figures 8 C-F**). This weak signal is considered to be the baseline expression of the Hsp 60 protein which is expressed by every mammalian cell.

During the bell stage, the Hsp 60 signal was strong in the inner enamel epithelium (IEE), outer enamel epithelium (OEE), presecretory ameloblasts (PreSecA), secretory ameloblasts (SecA), and stratum intermedium (SI) (E16.5-E18.5; **Figure 9**). Extracellular Hsp 60 signals were also found in the stratum intermedium. The immunoreactivity was increased in the cytoplasm of the preodontoblasts (PreO) and odontoblast (O) cells at E16.5.

Figure 8. The expression pattern of Hsp 60 in early stages of lower incisor development in mice

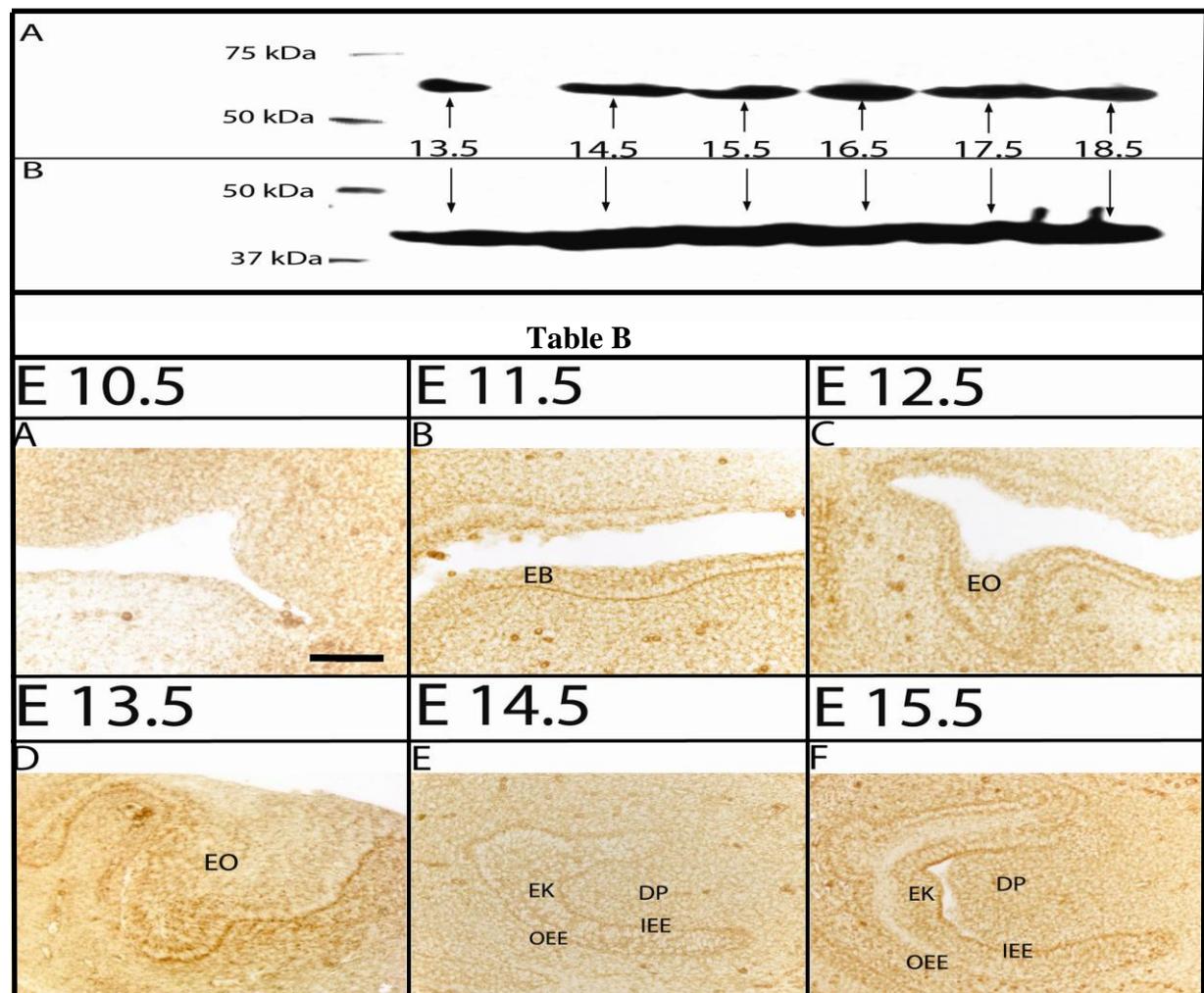
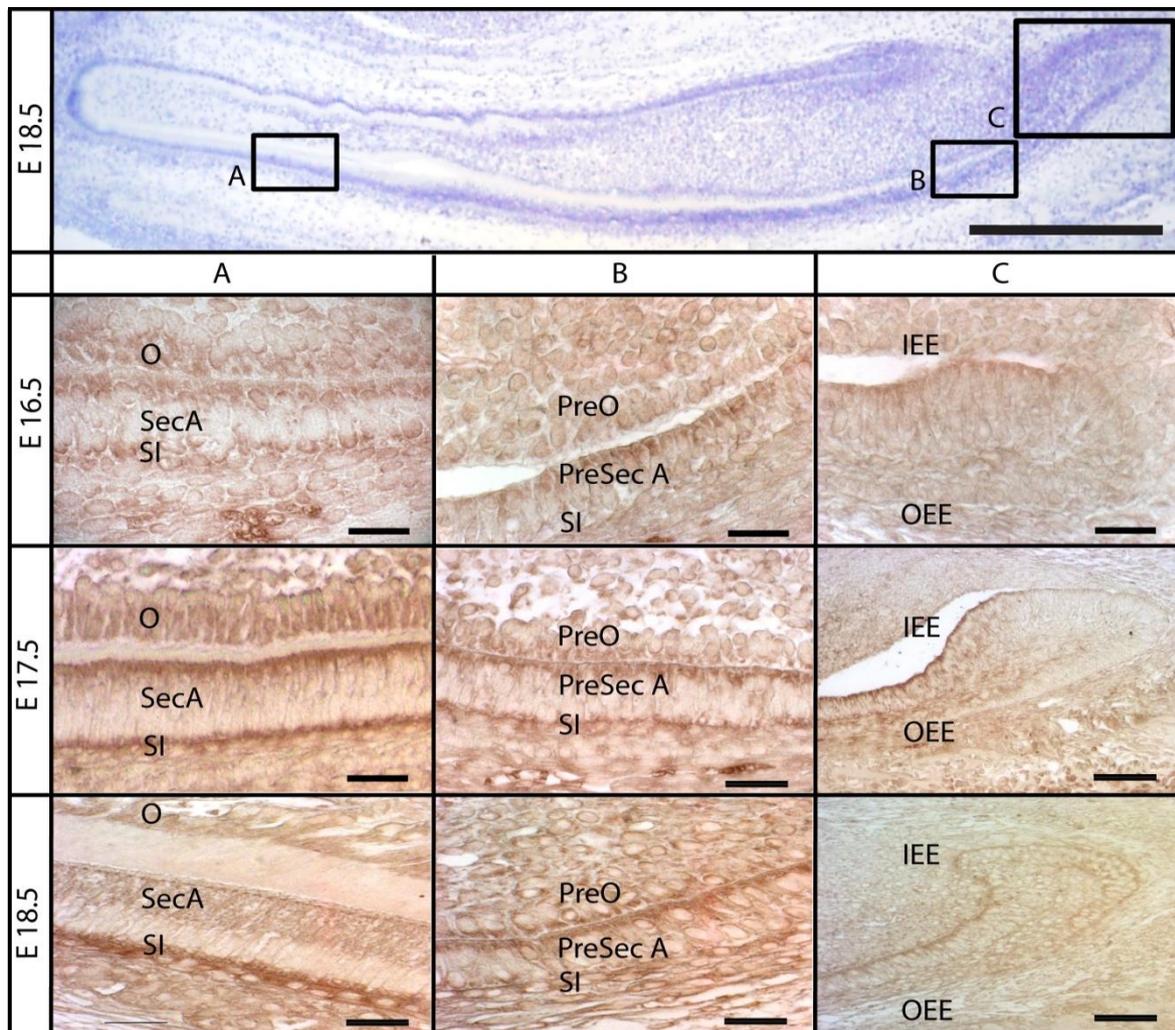


Table A; **A inset** shows the expression of Hsp 60 in the tooth germs from the bud stages of tooth development. **B inset** indicates the loading control. **Table B;** **A, F inset** shows the present of the Hsp 60 in the tooth germs from E13.5 to E15.5. Hsp 60 is present in the oral epithelium and epithelial band (EB) during the initial stage of the tooth development (**A-B inset**). From E12.5 to E15.5 (**C-F pictures**), Hsp 60 protein is present in the structures of the developing enamel organ (EO). From E14.5 (**D**), the inner enamel epithelium (IEE), outer enamel epithelium (OEE) and enamel knot (EK) of the enamel organ were positive for Hsp 60. The cells of dental papilla (DP) show weak Hsp 60 expression. Scale bar: A-D: 50 μ m, E-F: 100 μ m

Figure 9. The expression pattern of Hsp 60 during the bell stage of lower incisor development in mice

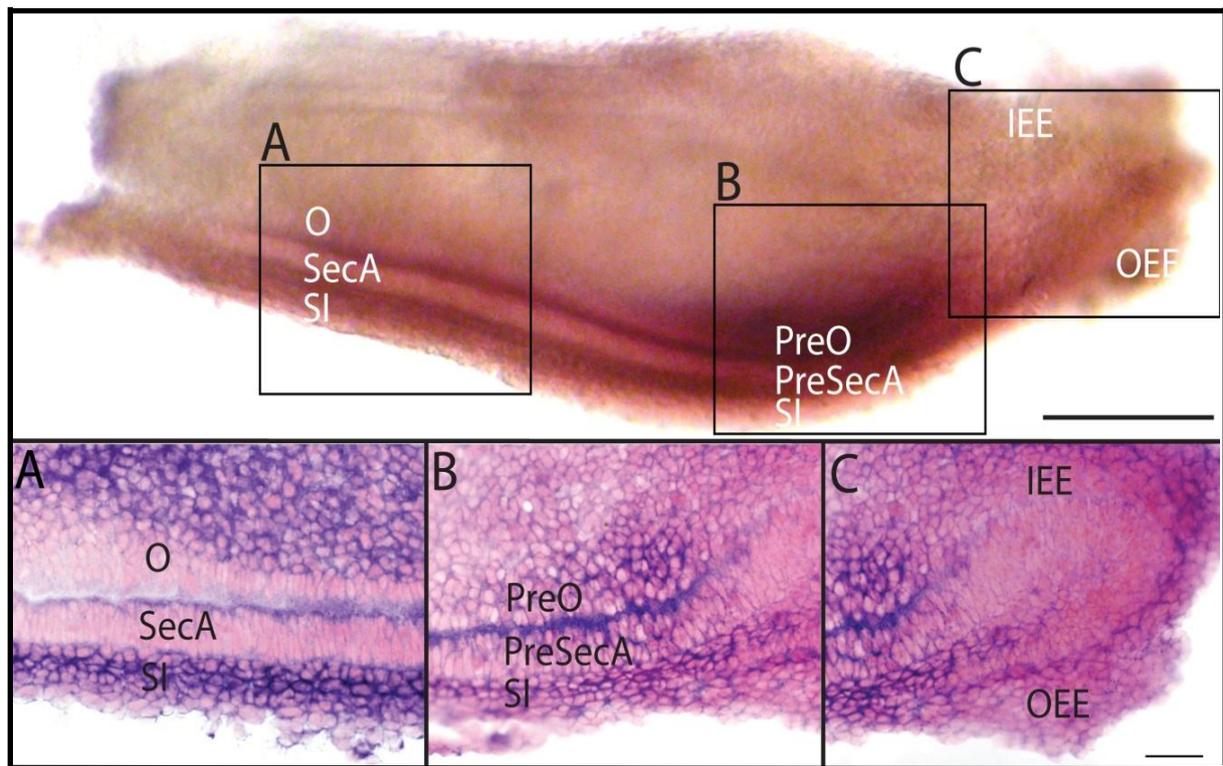


Insets in **Figure 9** show the localisation of the microphotos on histological slides. Column A shows the apical part of the enamel organ, B shows the proximal part of the enamel organ, and column C shows the labial root sheet. Presecretory ameloblasts (PreSecA), secretory ameloblasts (SecA), stratum intermedium (SI), inner enamel epithelium (IEE) and outer enamel epithelium (OEE) abundantly contain Hsp 60 signals. Preodontoblasts (PreO) and odontoblasts (O) also showed intensive immunoreaction. From E17.5, the stratum intermedium showed intensive Hsp 60 immunolabelling. Scale bar: A: 500 μm ; B-F, H,I: 20 μm ; G,J:100 μm .

5.6 The mRNA of Hsp 60 confirms the result of immunohistochemistry

Our goal was to reinforce the result of immunohistochemistry and detect the cellular origin of extracellular Hsp 60 which was present in stratum intermedium at E16.5. To reach this goal we detected the expression pattern of Hspd1 gene (mRNA of Hsp 60) by *in situ* hybridization. We see in the whole mount sample labelled strong Hspd1 expression in the labial part of enamel organ (**Figure 10**). The territory of the presecretory ameloblasts (PreSecA) and preodontoblasts (PreO) contain high concentration of Hspd1. The weak background in the dental papilla could be a basic expression of Hspd1. However strong signal was observed in the enamel organ according to the histological sections the distribution pattern is uneven in the samples. The **A, B, C** territory of whole mount labelling the positions of the histological sections, which were presented in higher magnification. Specially the outer enamel epithelium (OEE), inner enamel epithelium (IEE), presecretory ameloblasts (PreSecA), secretory ameloblasts (SecA), stratum intermedium (SI), preodontoblasts (PreO) and odontoblasts (O) showed strong positive reaction (**A; B; C insets**). The majority of Hspd1 situated in the cytosol of the apical part of dental cells. The lingual side of the tooth germ showed weak Hsp 60 mRNA expression. Interestingly the enamel knot was not labelled by the *in situ* hybridization (**Figure 10**). We can conclude that, the result of *in situ* hybridization correlates with the result of immunohistochemistry and the extracellular Hsp 60 originated from the cells of stratum intermedium (**Figure 10**).

Figure 10. Results of *in situ* hybridisation on E16.5 day-old tooth germ.



The whole mount sample showed the asymmetric distribution pattern of Hsp 60 in the tooth germ. The labial part contains strong signal against Hsp mRNA. **Insets A, B C** shows the labelled part of the whole mount sample in higher magnification after histological procession. The probe positively labelled the presecretory ameloblasts (PreSecA), secretory ameloblasts (SecA), preodontoblasts (PreO), odontoblasts (O), stratum intermedium (SI), outer enamel epithelium (OEE) of the tooth germ. Scale bar: 100 μ m, A-C insets: 20 μ m.

5.7 Exogenous Hsp 60 alters the morphology of the tooth germs

To investigate the possible effects of extracellular Hsp 60 on tooth germs, we used tooth germs in *ex vivo* organotypic culture in the presence of Hsp 60. The bell stage was chosen because this time point contains the morphological stage of the tooth development and this is the starting point of the histodifferentiation part of tooth development. Another argument to choose this stage, that the mineralization of the enamel may inhibit physically the uptake of exogenous Hsp 60. During the first day of culturing we did not detect any morphological difference between the Hsp 60 treated and non-treated tooth germs (**Figure 11 A-B**). Altered morphology in treated cultures was observed at the third day of culturing. The apical part of the treated tooth germs became blunted in shape whereas the proximal part of the tooth germ did not show any visual difference (**Figures 11 C-D**). More profound morphological changes between the treated and control cultures were observed at the fifth day of culturing. The distal parts were sharp in the control explants and blunted in the treated explants. The proximal part of the tooth germ showed no visual differences (**Figure 11 E-F**). Significant morphological alteration was detected between the treated and control groups (Mann-Whitney test, $p > 0.05$). Between the lingual and labial loops (Figure 5, E-F) significantly higher degree was closed by in treated samples ($21,01^\circ$; SD:3,77; SEM:1,68), than control samples ($11,88^\circ$; SD:2,94; SEM:1,31). This result enhances the macroscopic observations (Figures 5, G-H). We performed picosirius staining to identify the morphology of the explants. Sections were oriented parallel to the longitudinal axis of the tooth germs. Distal parts of the treated tooth germs were blunt in comparison to the control samples (**Figures 11 G-H**), confirming the observed macroscopic morphology.

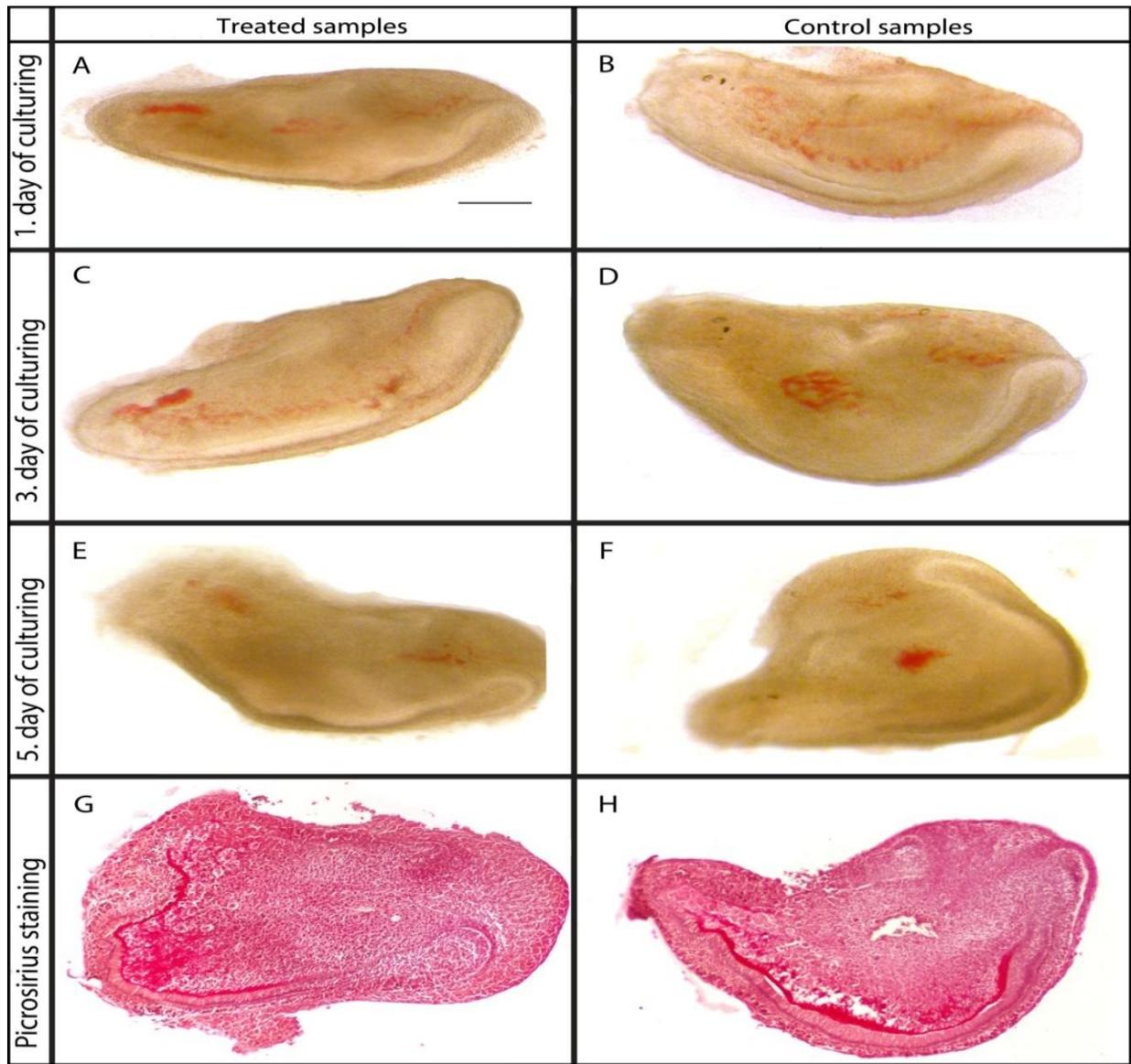


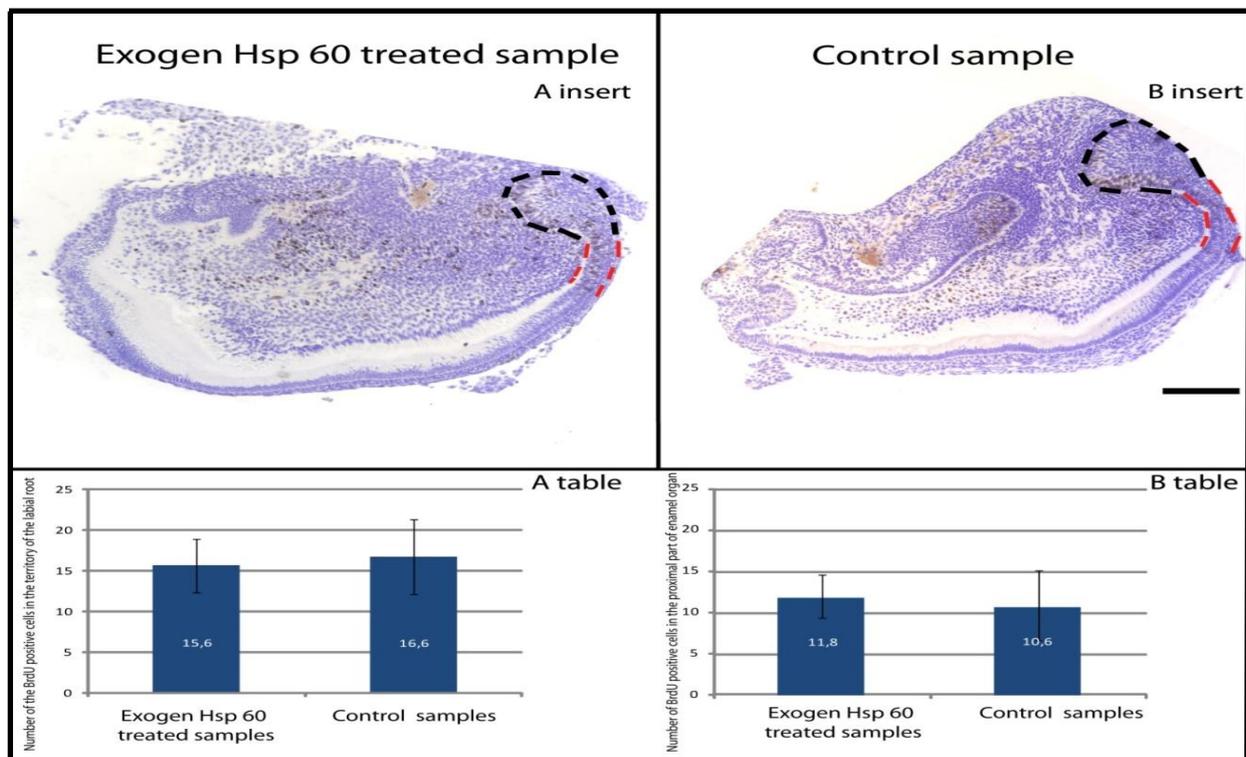
Figure 11. Exogenous Hsp 60 caused abnormal morphology of treated lower incisors

After the first day of culturing, no visual alteration was observed between the treated (A) and controls (B) explants. On the 5th culturing day, clear morphological differences were visible between the samples. The treated tooth germs (E) had blunted distal parts while the control samples had sharp distal parts (F). After the histochemical procession of the samples, more obvious differences developed between the two groups. The treated tooth germs had abnormal blunted distal parts (G) while the control sample had normal morphology (H). (Scale bar: A-F: 100 μ m, G-H: 50 μ m.)

5.8 Cell cycle analysis

The apical parts of the samples did not contain any dividing cells in the treated and control groups (**Figure 12**). Several BrdU positive cells were observed in the labial roots and in the proximal part of enamel organ in both groups. Although, distribution of the BrdU positive cells seemed slightly different in the two experimental group; we did not find any significant differences between the numbers proliferating cells (Mann-Whitney test, $p>0.84$). According to our results, Hsp 60 does not influence the cell cycle in the labial root of the 16.5 day-old tooth germs.

Figure 12. Hsp 60 treatment does not affect the proliferation rate of dental cells.



The exogenous Hsp 60 does not influence the dividing capacity of dental cells (**Figure 12 A and B table**). **A, B** inset indicate the territory of the labial root with black dashed line, the red dashed line labelled the territory of the investigated area of enamel organ. **A** table showed the number of the dividing cells in the territory of labial loop, **B** table showed the number of the dividing cells in the proximal part of enamel organ.

6. CONCLUDING REMARKS

6.1 Effects of Toll-like receptor 4 during tooth development

We described that TLR4 expressed from the cap stage (from E 14.5) and the expression later is continuous during the murine tooth development, which can indicate the developmental aspect of TLR4 during odontogenesis. Lipopolysaccharide, a potent agonist might cause the significantly decreased amount of I κ B during the *in vitro* culturing at the case of E16.5 tooth germs, which indicates the functional activity of TLR4 complex during the early stages of the tooth development, since the I κ B has major function through masking the nuclear translocation sequence of NF- κ B molecule, which is a major effector molecule of the downstream signaling of TLR4 (Hayden, 2008; Israel, 2010; Barak, 2014).

One of the most prominent effects of TLR4 is the modification of mineralization of hard tissues according to earlier findings (He et al, 2012; Johnson et al, 2004). To monitor this possible effect of TLR4 on tooth germ, we performed *in vitro* culturing where we applied LPS to the medium in order to activate TLR4. The explants of *in vitro* culturing we investigated by alizarin-red staining and ELISA against type X collagen on the treated and control samples. Comparing the mineralization status of samples by alizarin red staining, we can declare that LPS treatment decreases dramatically the amount of Ca⁺⁺ in the enamel at E16.5 day-old tooth germs in the 5 days of culturing. These finding overlaps with the result of previous study (He et al, 2012). Decreased mineralization was also observed during osteogenesis related to the TLR4 activation investigated by alizarin red-staining and ALP assay (Manoj et al, 2014; Chen et al, 2014). However weak mineralization (indicated by red color Figure 6) which can also be observed in the labial part of treated samples, can be the result of *in utero* mineralization.

We concern two possible answers to this alternation; first of all the downstream signaling pathway of TLR4 can decrease the ALP activity of dental cells, as previously described on osteoblasts (Vijayan V et al, 2014). Several studies investigate the role of TLR4 receptor signaling related to mineralization processes of dentin (Roberts et al, 2008; Mochida et al, 2009). According to these articles the LPS can inhibit the mineralization of hard tissues, through the modification of non-tissue specific alkaline phosphatase (Roberts et al, 2008; Mochida et al, 2009; He et al, 2012). Enhancing this, measured low alkaline phosphatase activity in the tooth germ related to LPS treatment. As another opportunity, LPS can bind free Ca^{++} , which connection can inhibit the incorporation of mineral phase into the dentin and enamel matrix (Mochida et al, 2009; Roberts et al, 2008; Leonardi et al, 2004). This reason was traversed by the result of *in situ* hybridization and RT-qPCR against ameloblastin mRNA, where we found acceleration of the maturing of ameloblasts (Wöltgens et al, 1995).

Underlining the results of alizarin-red staining and ALP activity related to mineralization, significantly decreased amount of collagen type X was measured by ELISA. Type X collagen has a principal role during the enchondral ossification providing the optimal environment of the hydroxide-apatite crystals (Shen G., 2005; Mackie et al, 2008). During tooth development type X collagen presents exclusively in the enamel matrix (Felszeghy et al, 2000). The lower level of type X collagen also indicates the weaker mineralization of enamel matrix (Felszeghy et al, 2000).

During the *in vitro* culturing of tooth germs we used biotin conjugated LPS. Concerning that LPS is specific agonists of TLR4, the structures which contain LPS have to express functionally active Toll-like receptor 4. The stratum intermedium and secretory ameloblasts contain the highest amount of biotin labelled LPS. These structures normally contain the major part of ALP, which control the enzymatic steps of the biomineralization of the tooth germ (Wöltgens et al, 1995).

Lower incisors of 16,5 day-old NMRI embryos has been used *in vitro* culturing to investigate ameloblastin expression level at the end of culturing, in order the screen the effects of LPS on secretory ameloblasts (Robinson et al, 1998). According to earlier studies, the enamel matrix proteins (amelogenins and non-amelogenins) are already present at E16,5 which indicating the early steps of enamel matrix synthesis. Ameloblastin concentration can affects the mineralization processes and expression level of it shows a negative correlation with the biomineralization proces of the enamel matrix (Teepe et al, 2014).

The ameloblastin is an indicator of ameloblast maturing, however transiently it is expressed in the dentin matrix (Satoshi et al, 2004; Simmer et al, 2010). *In situ* hybridization was performed to detect the expression pattern of ameloblastin mRNA at E16.5. Since AMBN was expressed only in the enamel organ, the whole amount of AMBN originated from ameloblasts. The treated tooth germs show accelerated ameloblastin mRNA synthesis (RT-qPCR), which indicates the role of TLR4 during differentiation of ameloblasts. Earlier findings describe the same effect of TLR4 on odontoblasts, namely that TLR4 activation increases the synthesis of matrix proteins of the dentin (decorin), and promote differentiation of odontoblasts (He et al, 2012; Stein et al, 2015). The accelerated ameloblast maturing can be the direct effect of the TLR4 signaling as well as on odontoblasts, or it can be compensatory mechanism of the inhibition of mineralization. To find an answer to this question we have investigated the possible role of MAPK signaling pathway, being is an open question during the tooth development, which is also activated by TLR4 (Zhang et al, 2001).

6.2 Expression pattern and possible role of Hsp 60 during odontogenesis

The second goal was to detect any possible endogenous ligand of TLR4, which can activate TLR4 under developmental processes. According to the literature several endogenous

ligands can activate the toll-like receptors, for example: Hsp-s, Gp96; HMGP; proteoglycans (Ohashi et al, 2000; Tsan et al, 2004; Laird et al, 2014; Fang et al, 2011; Stow et al, 2009; Nastase et al, 2012). From these candidates we choose the Hsp 60, because the Hsp 60 can activate or taken up by TLR4 receptor and it can modify the NF- κ B signaling pathway through the modification of IKK complex (Vabulas et al, 2002; Ohashi et al, 2000).

Earlier findings describe the expression patterns and possible roles of several heat shock proteins (Hsp 25, 27, Hsp 86, Hsc73, Hsj2) during tooth development. One of the most investigated heat shock protein is the Hsp 25 during the development of rat and mouse molars, which can take part in the morphological development and the differentiation of dental cells (Lee et al, 2009; Ohshima et al, 2000). The odontoblasts of murine tooth shows continuous Hsp 25 expression, while the dental pulp, preodontoblasts and ameloblasts only transiently express this protein (Ohshima et al, 2000).

Hsp 27 also take part in the morphological development of tooth germ, which is expressed in the dental epithelium (Leonardi et al, 2004). The expression pattern of these proteins is similar to the Hsp 27, the inner enamel epithelium and enamel knots contain the member of this protein family during the bell stage (Wada et al, 2002). The expression of Hsp 60 is similar to the Hsp 25 and Hsp 27 during the tooth development. The enamel epithelium and the odontoblasts contain more intensive immunoreaction than the dental follicle and dental papilla. The *in situ* hybridization against mRNA of Hsp 60 is a more sensitive method to enhance the result of IHC. The whole mount sample shows also uneven concentration of Hsp 60 between the different parts of the tooth germ. The histological section from the whole mount sample labels the Hsp 60 mRNA in the enamel organ, which overlaps with the results of immunohistochemical reactions. Important to note, that we collect the samples under nearly physiological conditions, which indicate that the high amount of Hsp 60 protein is not the result of any harm to tooth germs.

However pathological conditions such as hypoxia and low pH can cause elevated level of Hsp 60 (Morimoto et al, 1993). Related to this, we investigate the effect of high concentration of Hsp 60 in *ex vivo* cultures from 16.5 day-old tooth germs. Since Hsp 60 KO animal is not viable, we mimic the effect of elevated level of Hsp 60. The exogenous Hsp 60 cause blunt apical part of the treated tooth germs, while the proximal part shows no alteration, when compared the control and treated samples.

This alteration can result the activation of NF- κ B signaling pathway. Recent experimental data describe that the free cytosolic Hsp 60 can attach to the IKK complex (Chun et al, 2010). IKK complex activates the I κ B which break down and the free NF- κ B can activate gene expression. IKK consists of three subunits: IKK α , IKK β and IKK γ (Zandi et al, 1997; Mikkola, 2009). Absence of IKK α cause similar blunted apical part of lower incisors of IKK α KO tooth phenotypes in mice (Wenjing et al, 2010; Fleischmannova et al, 2008). Hsp 60 can attach directly to IKK α which can modify the effects of this protein. The I κ B KO mouse has normal phenotype related to lower incisors; it also indicates the principal role of the IKK complex (Fleischmannova et al, 2008). Another possible answer to the abnormal morphology can be the modification of the ectodysplasin/TNF signaling pathway, which has main role in morphological development of ectodermal appendices (Mikkola, 2009, Fraser et al, 2013). The effector of ectodysplasin/TNF pathway is the NF- κ B. The ectodysplasin/TNF mutant mice have similar tooth phenotype what we found in the case of the Hsp 60 treated samples. Interestingly the downstream signaling under the IKK complex did not altered the morphological development of tooth. During the BrdU incorporation test, the mitogenic effect of Hsp 60 could not be confirmed in dental cells (Di Felice et al, 2005). However the number of proliferating cells is slightly different in the territory of the labial root and the distal part of the enamel organ, this changing is not significant.

6.3 Heat shock protein 60 can be a ligand of Toll-like receptor 4

Our data suggest that TLR4 may take a part during histodifferentiation part of the tooth development. The Toll-like receptor 4 is expressed from the cap stage of the tooth development continuously. According to our findings the TLR4 is present in the structures of enamel organ of the tooth germ and in preodontoblasts. The activation of TLR4 at E16.5 inhibits the mineralization process of hard tissues of the tooth, which correlates with earlier data. Another important effect of LPS treatment is that it increases the maturing of ameloblast according to the *in situ* hybridization and RT-qPCR. During our experiments we used an exogenous ligand of TLR4, however a cardinal question that which endogenous ligand(s) can activate this receptor. One of the candidates was the heat shock protein 60. The immunohistochemistry method detected the Hsp 60 protein (as a potent agonist of TLR4) in the enamel organ and in odontoblasts in higher concentration than in neighboring structures. The *in situ* hybridization against HSPD1 enhances the result of immunohistochemistry, namely several structures of enamel organ contains elevated amount of Hsp 60 mRNA.

If we compare the result of our experiments we find physical similarities between the expression pattern of these peptides, enhancing the connection between the TLR4 and Hsp 60. Basically both TLR4 and Hsp 60 can modify the NF- κ B pathway; however other signaling pathways also can activate the NF- κ B pathway (Hayden et al, 2008). On the other hand, one part of our experiments does not enhance the role of Hsp 60 related to TLR signaling. Concerning that LPS (specific exogenous ligand) and Hsp 60 (non specific endogenous ligand) caused different morphology of the tooth germs, they may not use the same downstream signaling pathway or effector molecule. The activation of TLR4 break down the I κ B level as we detect in our experiments and others authors described earlier. Experimental data indicate that I κ B KO mice do not show any tooth abnormality in phenotype, however we found that Hsp 60 cause blunted distal part. Based on this we can discuss that the Hsp 60 may

not activate Toll-like receptor 4, but it can take up Hsp 60 and the cytosolic protein can attach to the IKK complex (Vabulas et al, 2002). According to these remarks the Hsp 60 does not acts as a typical ligand of the TLR4. Also important to note, that Hsp 60 can activate another TLRs, which can cause abnormal development of the tooth.

7. SUMMARY

Our goal was to identify the presence and possible role of Toll-like receptor 4 signaling cascade under tooth development and find any endogenous ligand (Hsp 60) of it, which may take a part under the developmental processes. According to our finding we declare the followings:

- The TLR4 is expressed from the cap stage of the tooth development and is present in high concentration in the structures of enamel organ.
- The activation of it with LPS did not caused any morphological alternation, but LPS treatments decreased the mineralization of the tooth germ and accelerated the differentiation of the ameloblasts.
- A possible ligand (Hsp 60) is expressed from the first day of tooth development. However the enamel organ contained it in high concentration in later stages.
- The Hsp 60 caused abnormal blunt distal part of the tooth germ, but did not affect proliferation rate of the dental cells.
- As a conclusion we described the expression pattern of the Hsp 60 and TLR4, but according to the different effects of these molecules the exact signaling pathway is not clear in details.

ÖSSZEFOGLALÁS

Előzetesen célul tűztük ki, hogy kimutassuk a jelenlétét és lehetséges szerepét a Toll-like receptor 4-nek a fogfejlődés során, illetve hogy detektáljuk egy endogén ligandját (Hsp 60) ennek a receptornak, melyek feltételezhetően szerepet játszik fejlődéstani folyamatokban. Eredményeink alapján az alábbiakat kijelenthetjük:

-A TLR4 a fogfejlődés során a sapka stádiumtól kifejeződik, legnagyobb mennyiségben a zománcszerv struktúráiban.

-A TLR4 aktiválása LPS-sel nem okozott morfológiai eltérést, de csökkentette a mineralizáció mértékét, illetve az ameloblasztok érését elősegítette.

-A lehetséges ligand (Hsp 60) a fogfejlődés első napjától kifejeződik, habár a zománcszerveben található nagy mennyiségben.

- A Hsp 60 abnormális tompa csúcsi részt okozott a fogfejlődés során, azonban a sejtek osztódását nem befolyásolta.

- Összefoglalva elmondhatjuk, hogy sikerült leírni a Hsp 60 és TLR4 kifejeződési mintázatát, de az eltérő hatásaik alapján a pontos jelátviteli útvonal feltérképezése további vizsgálatokat igényelnek.

8. KEY WORDS

tooth development, enamel organ, Toll-like receptor, heat-shock protein 60, IKK complex, mineralization, ameloblasts

fogfejlődés, zománcszerv, Toll-like receptor, hősokk fehérje 60, IKK komplex, mineralizáció, ameloblasztok

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11. APPENDICES



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Registry number: DEENK/2/2016.PL
Subject: Ph.D. List of Publications

Candidate: Tamás Papp
Neptun ID: FY4L4R
Doctoral School: Doctoral School of Dental Sciences
MTMT ID: 10037441

List of publications related to the dissertation

1. **Papp, T.**, Polyák, A., Papp, K., Mészár, Z., Zákány, R., Mészár-Katona, É., Terdik, T., Chang, H.H., Felszeghy, S.: Modification of tooth development by heat shock protein 60.
Int. J. Oral. Sci. "Accepted by Publisher" (2016), p. 1-8.
IF:2.531 (2014)
2. **Papp, T.**, Holló, K., Mészár-Katona, É., Nagy, Z., Polyák, A., Mikó, E., Bai, P., Felszeghy, S.: TLR signalling can modify the mineralization of tooth germ.
Acta Odontol. Scand. "Accepted by Publisher" (2016), p. 1-7.
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List of other publications

3. Albert, R., Vásárhelyi, G., Bodó, G., Kenyeres, A., Wolf, E., **Papp, T.**, Terdik, T., Módis, L., Felszeghy, S.: Computer-assisted microscopic analysis of bone tissue developed inside a polyactive polymer implanted into an equine articular surface.
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Total IF of journals (all publications): 5,842

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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