Modification of tooth development by heat shock protein 60

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RUNNING TITLE

The role of Hsp 60 in mouse tooth incisor development

Abstract

Since several heat shock proteins were investigated during tooth development, there is no available information about the spatial and temporal expression pattern of heat shock protein 60 (Hsp 60). To characterise Hsp 60 expression in the structures of the developing tooth germ, we used western blotting, immunohistochemistry and *in situ* hybridisation. Hsp 60 was present in high amounts in the inner and outer enamel epithelia, enamel knot, stratum intermedium and also appeared in odontoblasts beginning at the bell stage. To obtain functional data on the possible effect of Hsp 60 on isolated mouse lower incisors, we performed *in vitro* culturing. To investigate the effect of exogenous Hsp 60 on the cell cycle during culturing, we used the BrdU incorporation test on dental cells. Exogenously administered Hsp 60 caused bluntness at the apical part of the 16.5 day-old tooth germs, but it did not influence the proliferation rate of dental cells. We identified the expression of Hsp 60 in the developing tooth germ, which was present in high concentrations in the inner and outer enamel epithelia, enamel knot, stratum intermedium and odontoblasts. High concentration of exogenous Hsp 60 can cause abnormal morphology of the tooth germ, but it did not influence the proliferation rate of the dental cells. Our results suggest that increase level of Hsp 60 may cause abnormalities in the morphological development of the tooth germs and support the data on the significance of heat shock proteins during developmental processes.

Key words: Hsp 60, IKK, tooth development, morphology, enamel organ, mouse

Introduction

Tooth development is carried out and regulated by sequential and reciprocal interactions between the epithelial and neural-crest derived ectomesenchymal tissues (1-2). These processes are required for the precise temporal and spatial control of the cell cycle and cell differentiation (3). Interactions of several conserved signal transduction pathways, including those mediated by BMP (bone morphogenetic protein), Notch, Wnt (Wingless-related integration site protein), TNF (tumour necrosis factor), FGF (fibroblast growth factor), and SHH (sonic hedgehog) proteins (4-8), play key roles in coordinating and mediating this epithelial-mesenchymal cross-talk. Based on its properties the tooth germ is a very suitable tool to investigate developmental processes. Tooth development has three principal stages consisting of the initial, morphogenesis and histodifferentation stages (9). The epithelial-derived enamel organ is composed of the inner enamel epithelium, outer enamel epithelium, stratum intermedium, stellate reticulum, ameloblasts and enamel knot (10). The key structure of the cuspal morphogenesis is the enamel knot, which serves as a transient signalling centre during the morphogenetic stage of tooth development (11-12). The enamel knot appears during the cap stage, and stimulates the morphogenesis of the tooth germ indirectly via SHH and TNF signalling (13-14). The newly formed enamel organ is bounded by ectomesenchymal tissue, which forms the dental papilla, odontoblasts and dental follicle (13). The epithelial-derived cells of the tooth germ contain TNF receptors, which can be activated by ectodysplasin (15). The main role of the ectodysplasin/TNF signalling pathway during tooth development is the regulation of cuspal morphogenesis (16). TNF receptor activates IKK (inhibitor of κB kinase), which causes degradation of IκB (inhibitor of κB). In resting cells, the NF-κB (nuclear factor-κB) transcriptional factor forms a complex with IκB, which inhibits the nuclear translocation of NF-κB (17). In the absence of IκB, NF-κB is able to enter the nucleus where it modifies the transcription of various target genes (18).

This NF-κB pathway can be modified by many intra- and extracellular signalling molecules including heat shock protein 60 (Hsp 60) (19). Moreover, in a screen of the Hsp 60 expression profiles using online database (Allen Institute for Brain Science, Allen Developing Mouse Brain Atlas, available at http://developingmouse.brain-map.org), we found that Hsp 60 expression levels changed during different stages of tooth germs which was presumed a possible role of this molecule during development of the tooth. The family of Hsp (heat shock protein) includes several highly conserved proteins, which are expressed in every eukaryotic cell; and can be differentiated by their molecular weights and cellular localisation (20-21). Nearly 100 members of the Hsp family have been reported to be related to developmental processes (22). Despite the abundant research reports on Hsp 60 protein during embryonic development, data are scarce regarding the role of this molecule in the ontogeny of the tooth (22, 27). Hsp 60 is mainly localised to the mitochondrial membrane but is also present in the cytoplasm and which can be secreted into the extracellular matrix at low level under healthy conditions (23). Mitochondrial and cytoplasmic Hsp 60 has multiple functions. One of them includes protein refolding being translocated into the mitochondria. It also plays role in the modification of the NF-κB signal transduction pathway (24). Hsp 60 may also correlate with the cell cycle, whereby it can increase the proliferation rate of epithelial cells (25). The expression of Hsp can be triggered to strongly increase within minutes by pathological conditions (hypoxia, heat shock and low pH), which could lead to Hsp proteins comprising 30-40% of the total intracellular protein content (26). Under these pathological conditions Hsp 60 can be secreted as a danger signal into the extracellular space, which can modify the immune response as well as cell signalling pathways (28-29). Concerning these data, the Hsp 60 can reach transiently high concentration in extracellular space and in blood, which can influence developmental processes. Thus, Hsp 60 is not only a housekeeping protein, but also an early response element of cells against stress.

Therefore in this study, first we examined the expression pattern of Hsp 60 during development of the tooth under healthy conditions and then we investigated the possible effects of the high Hsp 60 concentration during the early stage of tooth development. The applied exogenous Hsp 60 during our experiments could mimic the effect of pathological environment of the tooth during embryonic development, which can give valuable information to understand better several congenital tooth anomalies.

Materials and methods

Animal care

All procedures were approved by the Animal Care and Use Committee of the University of Debrecen, and the study followed the guidelines set by the committee (DE FSZ/2010/10).

Sampling and tissue processing for histochemistry and immunohistochemistry

Experiments were carried out on lower incisors of NMRI mice. The age of the embryos was estimated from the appearance of the vaginal plug (E0) and from their external features; during our experiments we used three different embryos from E10.5 to E 18.5. Samples were isolated and fixed immediately in Sainte Marie fixative (30), dehydrated in graded series of ethanol and embedded into paraffin at 54 °C. Serial sections of 5-7 μm thickness were made in the coronal plane (E10.5-E12.5) and the sagittal plane (E13.5-E18.5) and were processed for further histological analysis. The *in vitro* culture samples were processed in the same way.

Western Blot

We tested the quality of exogenous Hsp 60 protein (Abcam, Cambridge, UK) using a monoclonal anti-Hsp 60 antibody (Thermo Scientific, Rockford, IL, USA). To confirm the specificity of the Hsp 60 immunohistochemical reaction, WB experiments were carried out on isolated 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 μg/mL Gordox, 10 μg/mL leupeptin, 1 mM phenylmethylsulphonyl-fluoride, 5 mM benzamidine, and 10 μg/mL trypsin inhibitor. Finally, 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 electrophoreticaly 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) in 1:200 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.

Immunohistochemistry and histochemistry

Deparaffinised and rehydrated tissue sections were immunostained with anti-Hsp 60 antibody at 1:200 in PBS (Thermo Scientific, Rockford, IL, USA) and visualised with a Vectastain Elite ABC Kit (Vector Laboratories Ltd., Peterborough, UK) according to the manufacturer's protocol. The slides were preincubated in 1% horse serum in PBS for 30 min at 24 °C to prevent the possibility of non-specific binding. As secondary antibody anti-mouse Ig-G (Vector Laboratories Ltd., Peterborough, UK) at 1:400 in PBS was applied, overnight, at 4 °C. The control sections originate from the same incisor in each case, these sections were stained in the same way, but the primary antibody was omitted and replaced with PBS. No signal was recorded from control sections incubated with PBS instead of primary antibody. Sections were visualised with DAB (3, 3'-diaminobenzidine) for conventional light microscopy. Each individual case of DAB precipitation in Hsp 60 immunostained sections was independently evaluated by two researchers at 400x magnification. The histochemical sections from *in vitro* culturing were stained with picrosirius F3B as described in the literature (31). This staining labelled the collagen fibers and amplified the optical anisotropy of collagen (32).

***In situ* hybridisation**

Mice were deeply anaesthetised with sodium-pentobarbital (50 mg/kg), and embryos were immediately processed. Non-radioactive probes were generated and used according to the Roche protocol (Roche, Mannheim, Germany) and the Dig-labelled probes were obtained by *in vitro* transcription using PCR templates (7, 33-34). PCR primers were chosen for regions containing exons from 7 to 12 and the 3'UTR regions using the mouse HSPD1 mRNA sequence (GenBank accession No: NM_010477.4) 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 the primers were as follows:

- T3 flanked sense: 5’-ATTAACCCTCACTAAAGGTCCCTGCTCTTGAAATTGCT-3’,
- T7 flanked antisense: 5’-AATACGACTCACTATAGGCTCCACAGAAAGGCTGCTTC-3’

(Integrated DNA Technologies, Inc., Coralville, IO, USA). *In situ* hybridisation was carried out as described earlier (33-34).

Organotypic tooth germ culture, morphological analysis

Following the literature, we performed a Trowel-type culture of the E16.5 lower incisors (35). The 16.5-day old stage was chosen because this is the latest time point of the morphological stage during tooth development and because at later stages, the maturing enamel may inhibit the uptake of exogenous Hsp 60. During separation, both lower incisors were separated from the mandibles under a Nikon SMZ 1000 stereomicroscope (Nikon, Tokyo, Japan). The whole tooth was carefully isolated and cultured in Trowel-type organ cultures. We placed the tissues on 0.1-μm pore-size nucleopore filters (Sigma, St. Louis, MO, USA) supported by metal grids in a humidified atmosphere of 5 % CO_2 in air at 37 °C. From each jaw, one of the incisors was used as the treated explant and the other as its individual control. The culture medium consisted of DMEM (Dulbecco's modified Eagle's medium) (Gibco Brl, Gaithersburg, MD USA) supplemented with 15 % foetal bovine serum (Gibco Brl, Gaithersburg, MD, USA). Exogenous Hsp 60 was added into the medium in 1 μg/ml (Abcam, Cambridge, UK) on the first (onset of culturing) and on the third day of culturing. The half-time of Hsp 60 is 3.2-10 minutes under healthy conditions (19, 36), the applied time period was important to follow the possible morphological changes of tooth germs. The culture medium was changed on the third day of culturing, the medium of treated explants was supplied with Hsp 60 (1 μg/ml). The experiment was concluded on the fifth day. The data

of *in vitro* culturings based on four individual experiments. To determine morphological alternation of tooth germs, the angle between the labial and lingual root of the tooth germs at the level of the enamel knot has been measured in 5 days old tissue cultured samples in treated and in control groups (n=7-7). We used *in vitro* samples to avoid the unwanted side effects of dehydration during histology staining.

Cell cycle analysis

10 µl/mL BrdU (5-bromo-2-deoxyuridine) labelling reagent (Life Technologies, Carlsbad, CA, USA) was added to the culture medium for 2 hours prior to fixation. The *in vitro* culture samples were fixed immediately in Sainte-Marie's fixative, dehydrated in graded series of ethanol and embedded in paraffin. Serial sections were cut in the sagittal plane at 5-7 µm and processed for further histological analysis. The BrdU was immunodetected using the BrdU Detection Kit according to the manufacturer's protocol (Zymed, Carlsbad, CA, USA).

Data analysis and image capturing

Histological samples were examined by transmitted light microscopy (Nikon Eclipse E 800, Tokyo, Japan), and representative images were captured with an Olympus DP 70 digital camera (Olympus, Tokyo, Japan). Images were edited with Adobe Photoshop CS4 Software (Adobe Systems Inc., San Jose, CA, USA). Data of morphological analysis was measured with ImageJ 1.46 (National Institutes of Health, Maryland, USA). Statistical analysis was based on 7 of each control and treated samples from 4 independent experiments. Significance of numerical data was verified by Mann-Whitney U-test.

Results

Hsp 60 protein and mRNA are present in high levels in the structures of the enamel organ and in the odontoblasts

We used western blot to detect Hsp 60 protein in the tooth germs from E13.5 to E18.5 (Figure 1). A single band at approximately 60 kDa was observed for each stage, confirming the ubiquitous expression of this protein. The distribution pattern of Hsp 60 protein was studied by immunohistochemistry in tooth germ slides ranging from E11.5 to 18.5. The results showed continuous expression of Hsp 60 during the early stages of enamel organ development (Figure 2, 3). The first appearance of Hsp 60 was detected by a weak DAB signal in the epithelial band (EB) during the initial stage (E11.5) of the tooth development (Figures 2, B). During the bud and cap stage (E13.5-E15.5; Figures 2, C-F), the inner enamel epithelium (IEE) and outer enamel epithelium (OEE) and enamel knot (EK) of the enamel organ showed intense Hsp 60 immunoreactive signals. In contrast, the dental papilla (DP) and dental follicle (DF) were weakly labelled (Figures 2, C-F), which we considered to be the baseline expression of the Hsp 60 protein. During the bell stage, the Hsp 60 signal was also strong in the derivatives of the enamel organ, including the inner enamel epithelium (IEE), outer enamel epithelium (OEE), preameloblasts (PreA), ameloblasts (A), and stratum intermedium (SI) (E16.5-E18.5; Figure 3). Moreover, homogenous DAB precipitations were found in the stratum intermedium, which may indicate extracellular Hsp 60. The immunoreactivity was increased in the cytoplasm of the preodontoblast (PreO) and odontoblast (O) cells at E16.5. A weak signal could be detected in the dental follicle (DP) as well as in the surrounding mesenchymal tissue.

To enhance the result of IHC and to investigate the cellular origin of the extracellular Hsp 60 present in the SI at E16.5, we performed *in situ* hybridisation. The majority of the Hsp 60 mRNA was localised to the labial side of the tooth germs, confirming the

immunohistochemical results (Figure 4). A strong signal was observed in the cells of the outer enamel epithelium (OEE), inner enamel epithelium (IEE), preameloblasts (PreA), ameloblasts (A), stratum intermedium (SI), preodontoblasts (PreO) and odontoblasts (O) while the lingual side of the tooth germ showed weak mRNA expression (Figure 4).

Exogenous Hsp 60 alters the morphology of the tooth germs

To further investigate the possible effects of extracellular Hsp 60 on tooth development, *ex vivo* organotypic tooth germs were cultured in the presence of Hsp 60 administered to the culture medium. There were no detectable morphological differences during the first day of *in vitro* culturing between the Hsp 60 treated and non-treated tooth germs (Figure 5, A-B). Altered morphology in treated cultures was first observed at the 3rd DIV. The apical part of the treated tooth germs became blunted in shape with a clearly visible enamel knot whereas the proximal part of the tooth germ did not show any visual difference (Figures 5, C-D). More profound morphological changes between the treated and control cultures were observed at the 5^{th} day of culturing. The distal parts were sharp in the control explants and blunt in the treated explants. Similarly to the earlier time points, the proximal part of the tooth germ showed no visual differences (Figure 5, E-F). We performed picrosirius histochemical 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 5, G-H), confirming the observed macroscopic morphology described above with one important note: the altered side had a clear border and living cells, which was in contrast to our expectations based on macroscopic observations. According to morphological analysis significant alteration was detected between the treated and control groups (Mann-Whitney test, $p>0.05$). The lingual and labial loops (Figure 5, E-F) closed significantly higher degree in treated samples (21,01°; SD:3,77; SEM:1,68), than

control samples (11,88°; SD:2,94; SEM:1,31). This result correlates with the morphology of the histology observation (Figures 5, G-H).

Cell cycle analysis

We used the BrdU incorporation test to detect the dividing cells of the tooth germs. The apical parts of the samples did not contain any dividing cells in either the treated or control groups (Figure 6). Several BrdU positive cells were found in the labial roots and in the proximal part of the enamel organ in both groups. Although the distribution of BrdU positive cells seemed slightly different in the two experimental groups, we did not find any significant differences between the numbers and distribution of these 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 germ.

Discussion

To the best of our knowledge, this is the first study describing the expression pattern and possible effect of Hsp 60 during tooth development. It is important to note, that all of the heat shock proteins are essential components of the organisms, although they had diverse function and various expression patterns during the tooth development. During odontogenesis the expression patterns and possible roles of Hsp 25, Hsp 27, Hsp 86, Hsc73, Hsj2 were already described (37-41). Among these, the Hsp 25 has been studied the most in rat and mice incisors (37-38). The dental pulp, preodontoblasts and ameloblasts were transiently positive for Hsp 25, while the odontoblasts were showed continuous Hsp 25 expression (38). The Hsp 27 was detected in the dental epithelium of bell stage and it might be related to the morphological development of the tooth (41). The distribution pattern of Hsp 86, Hsj2 and Hsc73 were similar to each other in the enamel organ: the inner enamel epithelium and

primary enamel knots showed strong signals during the bell stage (40). According to our findings in Hsp 60, we found similar expression pattern with the Hsp 25 and Hsp 27: more intense immunoreaction were in the structures of the enamel epithelium and the odontoblasts than in the neighbouring structures. This observation was also confirmed by a more sensitive method, whole mount *in situ* hybridization against mRNA of Hsp 60.

The Hsp 60 has several intracellular functions under healthy conditions (23-26). In contrast less is known about the possible role of extracellular Hsp 60. In our culturing experiments extracellular Hsp 60 caused abnormal morphology, one of the possible candidates for the effectors mechanism is the TLR4 receptor signalling. Exogenous Hsp 60 can be taken up by TLR4 receptor, which can activate the NF-κB signalling pathway (42-43). In our study, excess Hsp 60 caused an abnormal morphology (Figure 5) of the distal part of the tooth germ. In these experiments, the applied high level of Hsp 60 during the *in vitro* culturing model we were aimed to mimic the increased levels of Hsp 60 which may originate from tissues of the tooth germ under pathological conditions, such as hypoxia, placental insufficiency, or chronic fever during pregnancy. Moreover, our preliminary experiments showed the present of TLR4 receptor in the tooth germs used by Western blot, which can strengthen our hypothesis for downstream acting of Hsp 60 though TLR4. Nevertheless, despite the large number of studies about the TLR4 signalling pathway on odontoblasts, we did not find any study related to the regulation of tooth development (44-45).

Another opportunity the ectodysplasin/TNF signalling pathway may also take part on the effect of the Hsp 60. This pathway has major role in the morphological development of ectodermal appendices, and the downstream portion of the signalling pathway includes the IKK complex (46). IKK consists of three subunits: IKKα, IKKβ and IKKγ (46) and plays an NF-κB-independent role during the early stage of embryonic development by influencing the invagination of the ectoderm-derived tooth germ and whiskers into the underlying

mesenchyme (48-49). Recent *in vitro* culture experiments support earlier findings in which free cytosolic Hsp 60 can attach to the IKK complex and the absence of IKKα can cause abnormal tooth phenotypes (19, 50-51). Similar morphology was observed between the treated incisor tooth germs and the lower incisors of IKKα KO mice (48). However, IKK modifies the degradation of IκB, resulting in normal tooth morphology in the IκB KO mice. This indicates that the blunted distal part of the treated tooth germs in our experiments probably is not the result of altered expression of NF-κB target genes (48). This background supports that the Hsp 60 may attach to IKKα and that this connection may inhibit the function of IKKα in developmental processes (19).

During the BrdU incorporation test, we investigated the labial root and the distal part of the tooth germ based on the expression pattern of Hsp 60 in the enamel organ. The labial root contains pluripotent stem cells, which give rise to the epithelial cells of the inner enamel epithelium (52-53). According to the literature, Hsp 60 may increase the proliferation rate of the epithelium-derived cells (54). However, this mitogenic effect of Hsp 60 could not be confirmed in dental cells. We also investigated the apical part of the tooth germs, which is responsible for forming the shape of the tooth (55). The enamel knot is a transient structure that is responsible for the final morphology of the tooth and does not contain proliferating cells in the bell stage (56). This was not altered by exogenous Hsp 60. Our results suggested that Hsp 60 does not modify the proliferation activity of the dental cells in the enamel organ and in the labial root.

Conclusion:

Here we presented a description on the expression pattern of Hsp 60 mRNA and protein during tooth development. According to our results this protein can play role in the morphological developmental of the tooth germ. Although the exact mechanism by which Hsp 60 influenced morphogenesis of tooth germs has not been clarified yet, but our results suggested the involvement of the IKK complex. This study supported the roles of heat shock proteins during developmental processes, concerning, that the elevated amount of Hsp 60 may be the result of pathological processes.

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Conflict of interest

The authors declare no conflict of interest.

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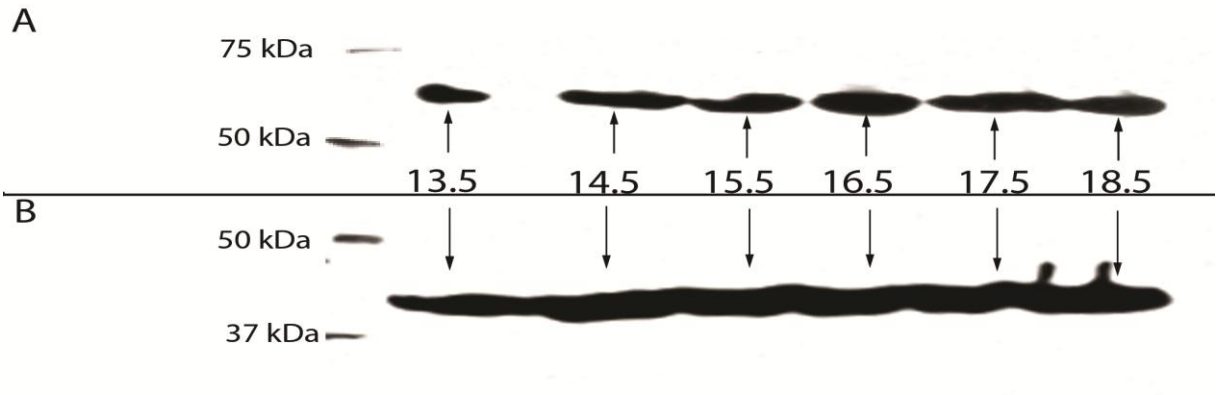
Figure legends

Figure 1

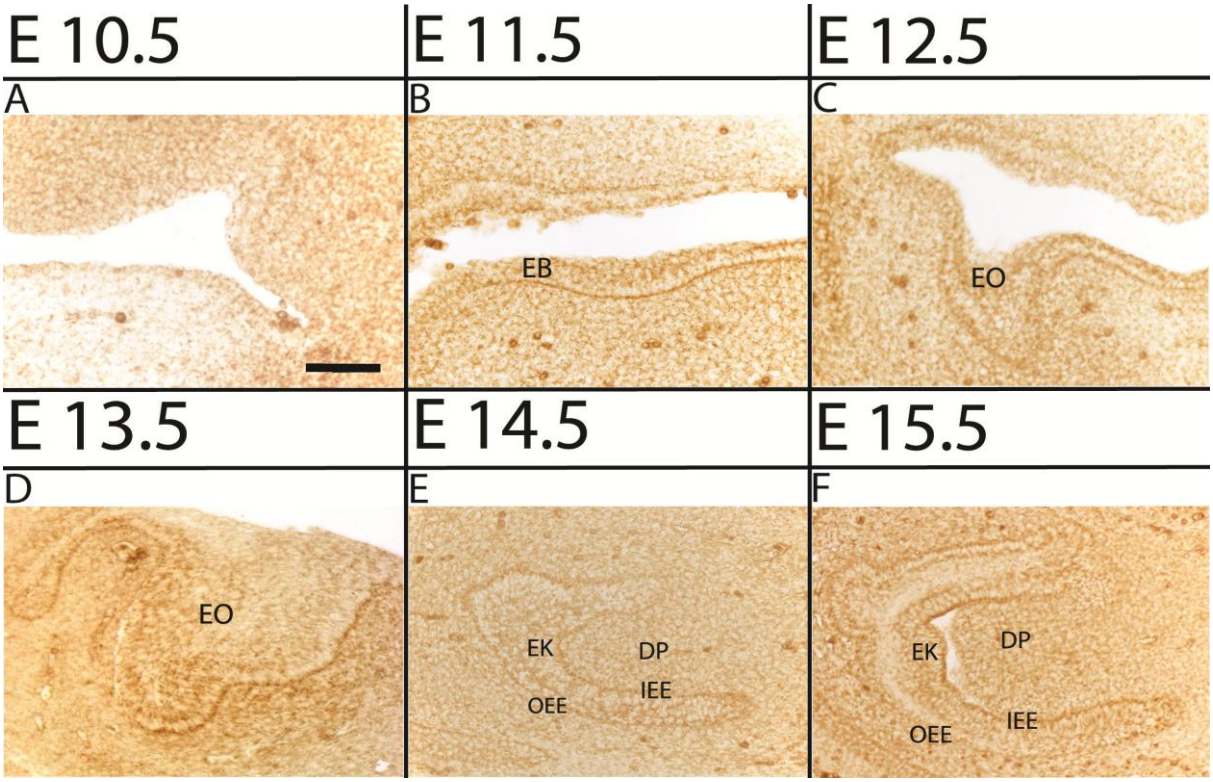
Results of Western blot analysis.



A table shows the presence of Hsp 60 in the tooth germs from E13.5 to E18.5. B table shows the loading control (actin).

Figure 2

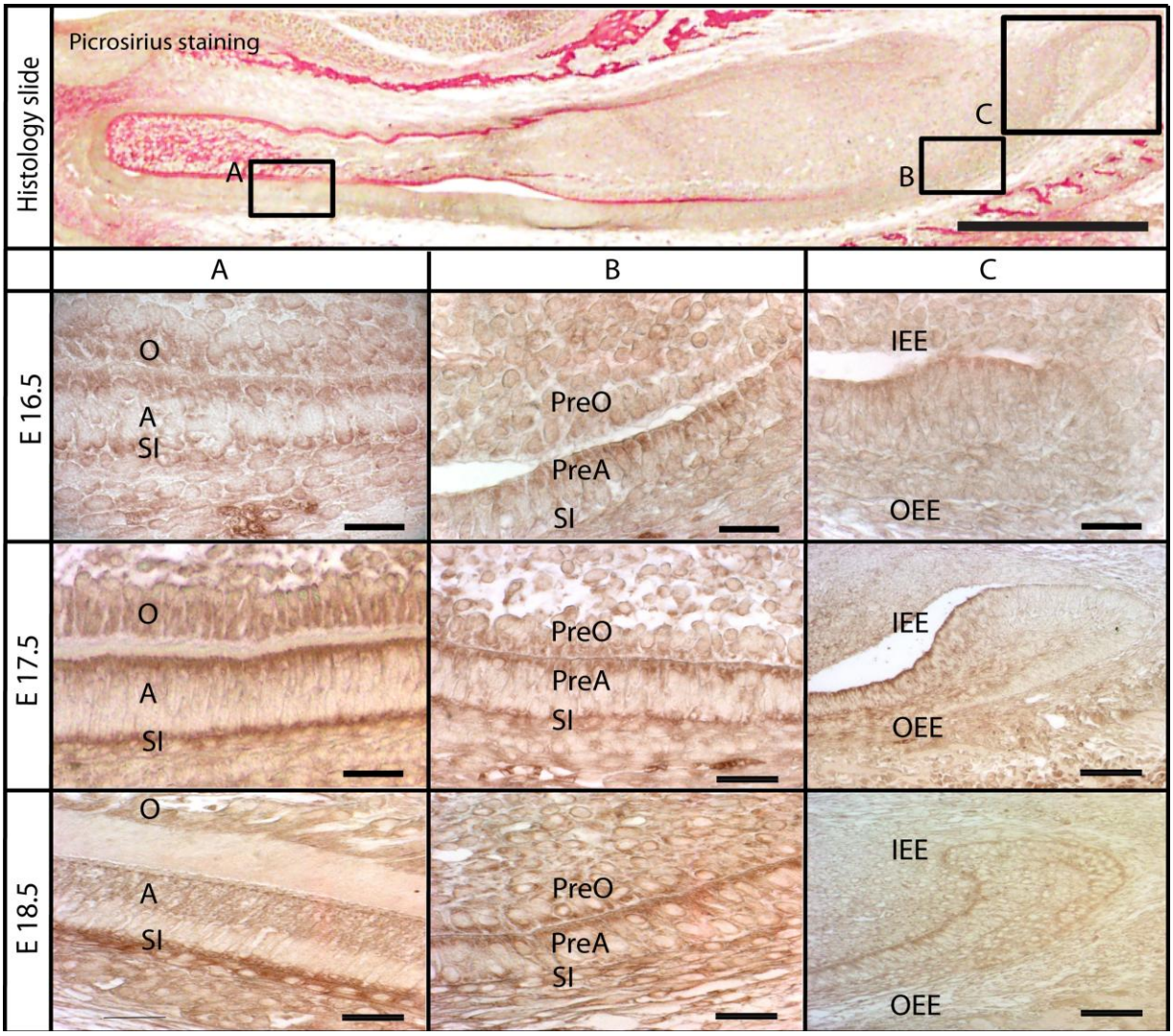
The expression pattern of Hsp 60 in early stages of lower incisor development in mice.



Hsp 60 is present in the oral epithelium and epithelial band (EB) in the initial stage of the tooth development (A-B insert). 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 µm, E-F: 100 µm

Figure 3

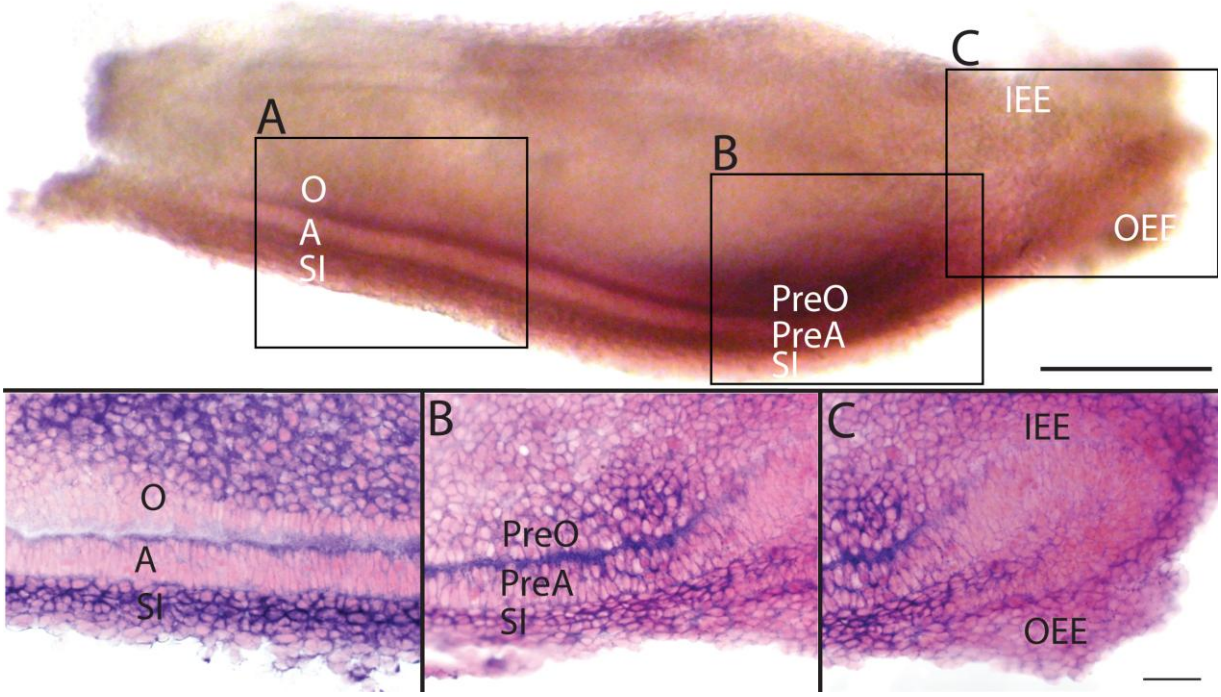
The expression pattern of Hsp 60 during the bell stage of lower incisor development.



Histology slide was stained by Picrosirius to help to recognize the position of inserts of A, B and C columns from different stages (E16.5-E18.5). 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. Preameloblasts (PreA), ameloblasts (A), stratum intermedium (SI), inner enamel epithelium (IEE) and outer enamel epithelium (OEE) abundantly contain Hsp 60 signals. Preodontoblasts (PreO) and odontoblasts (O) also show intensive immunoreaction. From E17.5, the stratum intermedium shows intensive Hsp 60 immunolabelling. Scale bar: A: 500 µm; B-F, H,I: 20 µm; G,J:100 µm.

Figure 4

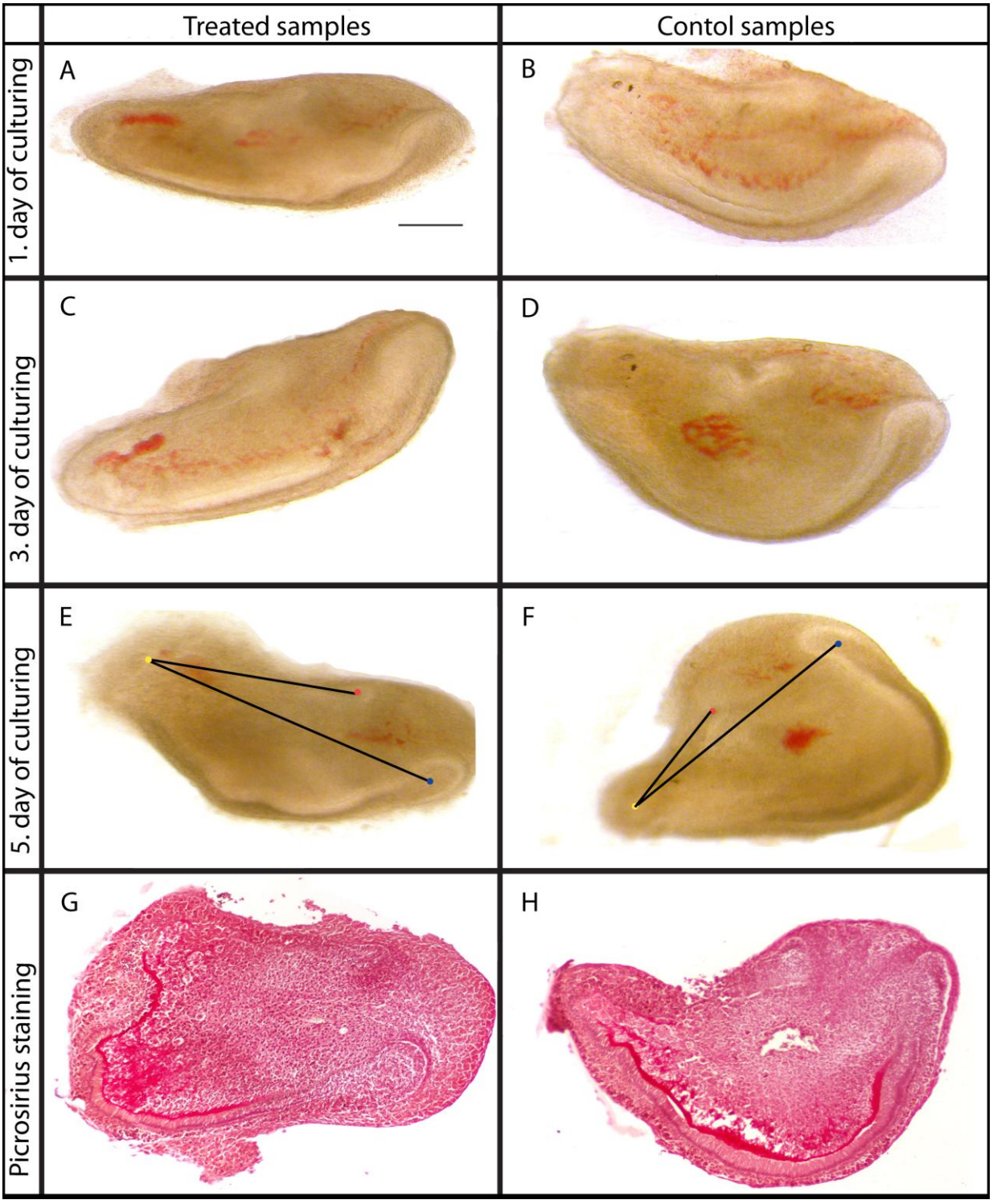
Results of *in situ* hybridisation on 16.5 day-old tooth germ.



To verify the results of the immunohistochemistry, we performed *in situ* hybridisation, which confirmed the enhanced expression of Hsp 60 in the enamel organ and in odontoblasts. Insert A shows the apical part of the enamel organ a higher magnification, the insert B shows the proximal part, insert C shows the labial root from the whole mount sample. The probe positively label the preameloblasts (PreA), ameloblasts (A), preodontoblasts (PreO), odontoblasts (O), stratum intermedium (SI), outer enamel epithelium (OEE) of the tooth germ. The inner enamel epithelium (IEE) also shows signal that is not very intense. Scale bar: 100 µm, A-C inserts: 20 µm.

Figure 5

Exogenous Hsp 60 causes abnormal morphology of the *in vivo* cultured E16.5 incisor.

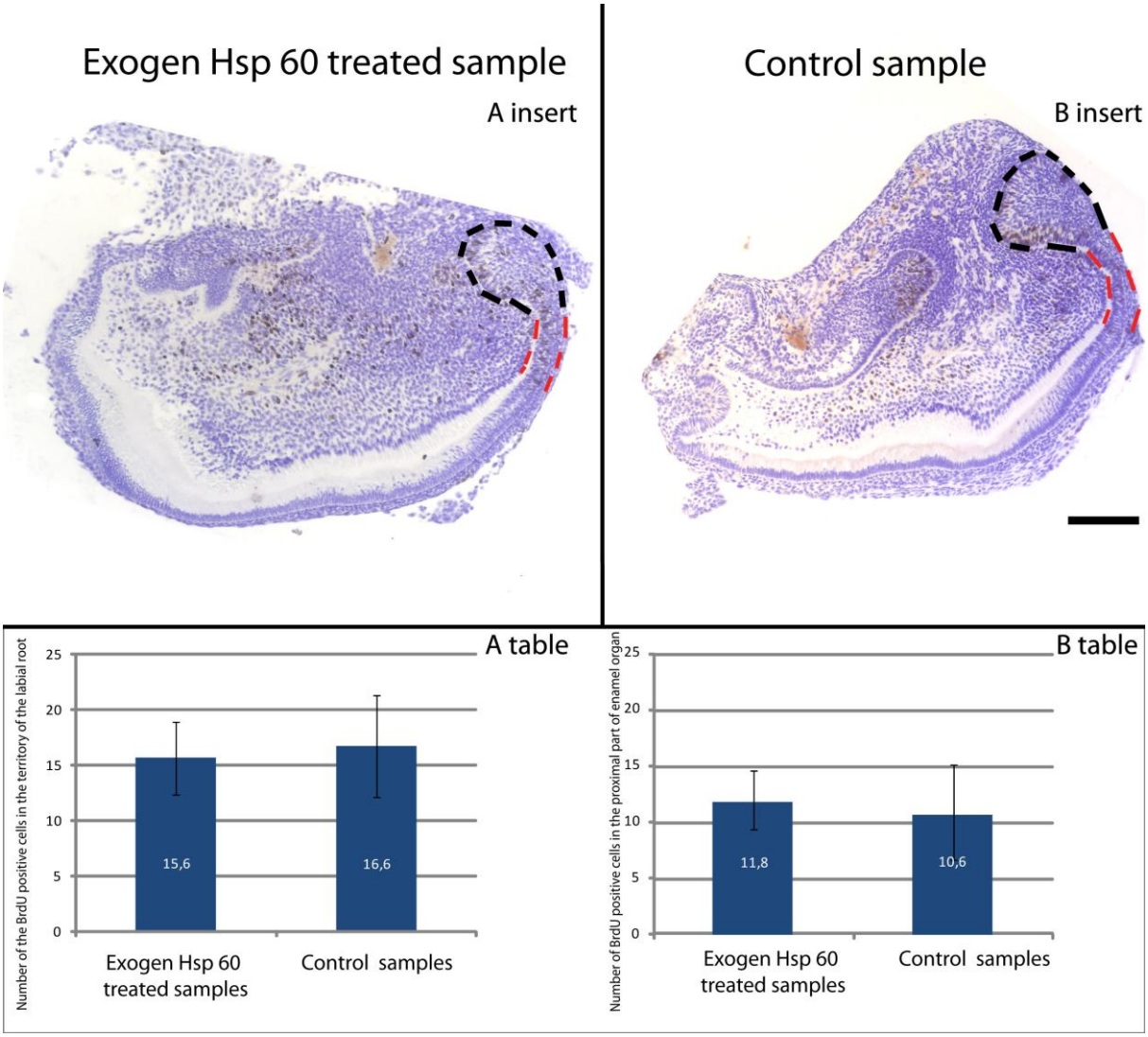


At the end of the first day in culture, no visual difference can observe between the treated (A) and control (B) tooth germs. The first morphological sign appears on the third day of *in vitro*

culture. The distal part of the treated samples (C) start to show blunted apical parts while the apical part of the control sample shows normal morphology (D). On the fifth culture day, clear morphological differences develop between our samples. The treated tooth germs (E) have blunted distal parts while the control samples have sharp distal parts (F). Red point indicates the centre of lingual loop, blue point indicates centre of labial loop and yellow point indicates the enamel knot (E-F inserts). The enclose degree between these points at the case of treated samples is significantly higher ($p>0.05$). After the histochemical staining, more obvious differences develop between the two groups. The treated tooth germs have abnormal blunted distal parts (G) while the control samples have normal morphology (H). The intense red colour indicates collagen in the predentin. (Scale bar: A-F: 100 μm, G-H: 50 μm.)

Figure 6

Effect of Hsp 60 on the proliferation of dental cells.



Proliferating cells incorporated BrdU are visible in brown colour. Hsp 60 does not modify the number of proliferating cells in the labial root (investigated territory bounded by black dashed lines on A-B inserts; A table) and in the enamel organ (investigated territory bounded by red dashed lines on A-B inserts; B table). The cell cycle analysis shows no difference between the viability of the dental cells in the treated and control groups, and the apical part of the tooth germs contains no proliferating cells. Though we detect several dividing cells in the territory of the enamel organ and labial root, the statistical analysis shows no significant difference between the numbers of BrdU-positive cells ($p>0.84$). (Scale bar: 100 μm.)