

## ORIGINAL ARTICLE

# Modification of tooth development by heat shock protein 60

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Although several heat shock proteins have been investigated in relation to tooth development, no available information is available about the spatial and temporal expression pattern of heat shock protein 60 (Hsp 60). To characterize Hsp 60 expression in the structures of the developing tooth germ, we used Western blotting, immunohistochemistry and *in situ* hybridization. Hsp 60 was present in high amounts in the inner and outer enamel epithelia, enamel knot (EK) and stratum intermedium (SI). Hsp 60 also appeared in odontoblasts beginning in the bell stage. To obtain data on the possible effect of Hsp 60 on isolated lower incisors from mice, we performed *in vitro* culturing. To investigate the effect of exogenous Hsp 60 on the cell cycle during culturing, we used the 5-bromo-2-deoxyuridine (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, EK, SI 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 increased levels of Hsp 60 may cause abnormalities in the morphological development of the tooth germ and support the data on the significance of Hsp during the developmental processes.

*International Journal of Oral Science* (2016) 8, 24–31; doi:10.1038/ijos.2015.53; published online 22 January 2016

**Keywords:** enamel organ; heat shock protein 60; inhibitor of  $\kappa$ B kinase; morphology; mouse; tooth development

## INTRODUCTION

Tooth development is carried out and regulated by sequential and reciprocal interactions between the epithelial and neural crest-derived ectomesenchymal tissues.<sup>1–2</sup> These processes are required for the precise temporal and spatial control of the cell cycle and cell differentiation.<sup>3</sup> Interactions of several conserved signal transduction pathways, including those mediated by bone morphogenetic protein (BMP), Notch, wntless-related integration site protein (Wnt), tumour necrosis factor (TNF), fibroblast growth factor (FGF) and sonic hedgehog (SHH) proteins,<sup>4–8</sup> play key roles in coordinating and mediating this epithelial–mesenchymal crosstalk. Based on its properties, the tooth germ is a very suitable tool to investigate developmental processes. Tooth development has three principal stages: the initial, morphogenesis and histodifferentiation stages.<sup>9</sup> The epithelial-derived enamel organ is composed of the inner enamel epithelium (IEE), outer enamel epithelium (OEE), stratum intermedium (SI), stellate reticulum, ameloblasts (A) and enamel knot (EK).<sup>10</sup> The key structure of cuspal morphogenesis is the EK, which serves as a transient signalling centre during the morphogenetic stage of tooth development.<sup>11–12</sup> The EK appears during the cap stage and stimulates the morphogenesis of the tooth germ indirectly *via* SHH and TNF signalling.<sup>13–14</sup> The newly formed enamel organ is bounded by ectomesenchymal tissue, which forms the dental papilla (DP), odontoblasts and dental follicle (DF).<sup>15</sup>

The epithelial-derived cells of the tooth germ contain TNF receptors, which can be activated by ectodysplasin.<sup>15</sup> The main role of the ectodysplasin/TNF signalling pathway during tooth development is the regulation of cuspal morphogenesis.<sup>16</sup> The TNF receptor activates the inhibitor of  $\kappa$ B kinase (IKK), which causes degradation of the inhibitor of  $\kappa$ B (I $\kappa$ B). In resting cells, the nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcriptional factor forms a complex with I $\kappa$ B, which inhibits the nuclear translocation of NF- $\kappa$ B.<sup>17</sup> In the absence of I $\kappa$ B, NF- $\kappa$ B is able to enter the nucleus where it modifies the transcription of various target genes.<sup>18</sup>

This NF- $\kappa$ B pathway can be modified by many intra- and extracellular signalling molecules including heat shock protein 60 (Hsp 60).<sup>19</sup> Moreover, in a screening of the Hsp 60 expression profiles using an 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 the different stages of the growth of the tooth germ, which was presumed to be a possible role of this molecule during development of the tooth. The family of Hsp includes several highly conserved proteins, which are expressed in every eukaryotic cell. These highly conserved proteins can be differentiated by their molecular weights and cellular localization.<sup>20–21</sup> Nearly 100 members of the Hsp family have been reported to be related to developmental processes.<sup>22</sup> Despite the

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Accepted 30 October 2015

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.<sup>22–23</sup> Hsp 60 is mainly localized to the mitochondrial membrane, but it is also present in the cytoplasm and can be secreted into the extracellular matrix at low levels under healthy conditions.<sup>24</sup> Mitochondrial and cytoplasmic Hsp 60 have multiple functions. One of these functions includes protein refolding being translocated into the mitochondria. Hsp 60 also plays a role in the modification of the NF- $\kappa$ B signal transduction pathway.<sup>25</sup> Hsp 60 may also correlate with the cell cycle, whereby it can increase the proliferation rate of epithelial cells.<sup>26</sup> 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.<sup>27</sup> 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.<sup>28–29</sup> 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 in stressful conditions.

Therefore, in this study, we first examined the expression pattern of Hsp 60 during the development of teeth under healthy conditions. Then, we investigated the possible effects of the high Hsp 60 concentration during the early stage of tooth development. The exogenous Hsp 60 applied during our experiments could mimic the effect of a pathological environment of teeth during embryonic development, which can give valuable information for better understanding several congenital teeth 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 E18.5. Samples were isolated and fixed immediately in Sainte-Marie's fixative,<sup>30</sup> dehydrated in a graded series of ethanol and embedded into paraffin at 54 °C. Serial sections of 5–7  $\mu$ m 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, Western blot experiments were carried out on isolated tooth germ from E13.5 to E18.5 stages. The tooth germ remained intact, and the surface of the tooth germ did not contain connective tissue. Isolated tooth germ was placed in 50  $\mu$ L homogenization buffer containing 50 mmol·L<sup>-1</sup> Tris-HCl buffer (pH 7.0), 10  $\mu$ g·mL<sup>-1</sup> Gordox, 10  $\mu$ g·mL<sup>-1</sup> leupeptin, 1 mmol·L<sup>-1</sup> phenylmethylsulphonyl-fluoride, 5 mmol·L<sup>-1</sup> benzamidine and 10  $\mu$ g·mL<sup>-1</sup> trypsin inhibitor. Finally, the tooth germ

was sonicated by pulsing bursts (Cole-Parmer, East Bunker Court Vernon Hills, IL, USA). For Western blot, total cell lysates were used. Samples for sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) were prepared by the addition of twofold concentrated electrophoresis sample buffer to cell lysates to equalize the protein concentration in samples, followed by boiling for 10 min. Then, 10–20  $\mu$ g of protein was separated by 7.5% SDS-PAGE for detection of Hsp 60 and actin. Proteins were electrophoretically transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS) for 1 h 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 3  $\times$  10 min in PBS with 0.05% Triton-X and 0.3 mol·L<sup>-1</sup> NaCl (PBST), membranes were incubated with anti-mouse IgG secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA) in 1:1 500 dilution for 1 h at room temperature. Signals were detected by enhanced chemiluminescence (Millipore, Temecula, CA, USA) according to the manufacturer's instructions. Signals were manually developed on an X-ray film.

### Immunohistochemistry and histochemistry

Deparaffinized and rehydrated tissue sections were immunostained with anti-Hsp 60 antibody at 1:200 in PBS (Thermo Scientific, Rockford, IL, USA) and visualized with a Vectastain Elite ABC Kit (Vector Laboratories, 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. Secondary antibody anti-mouse IgG (Vector Laboratories, Peterborough, UK) at 1:400 in PBS was applied, overnight, at 4 °C. The control sections originated from the same incisor. These control 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 visualized with 3,3'-diaminobenzidine (DAB) for conventional light microscopy. Each individual case of DAB precipitation in Hsp 60 immunostained sections was independently evaluated by two researchers at  $\times$ 400 magnification. The histochemical sections from *in vitro* culturing were stained with picosirius F3B as described in the literature.<sup>31</sup> This staining labelled the collagen fibres and amplified the optical anisotropy of collagen.<sup>32</sup>

### In situ hybridization

Mice were deeply anaesthetized with pentobarbital sodium (50 mg·kg<sup>-1</sup>), 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 polymerase chain reaction (PCR) templates.<sup>7,33–34</sup> 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'-ATTAACCCTCACTAAAGGTCCCTGCTC TTGAAATTGCT-3'

– T7 flanked antisense: 5'-AATACGACTCACTATAGGCTCCAC AGAAAGGCTGCTTC-3'

(Integrated DNA Technologies, Coralville, IA, USA). *In situ* hybridization was carried out as described earlier.<sup>33–34</sup>

### Organotypic tooth germ culture and morphological analysis

Following the literature, we performed a Trowel-type culture of the E16.5 lower incisors.<sup>35</sup> 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- $\mu\text{m}$  pore-size nucleopore filters (Sigma, St. Louis, MO, USA) supported by metal grids in a humidified atmosphere of 5%  $\text{CO}_2$  in air at 37 °C. From each jaw, one of the incisors was used as the treated explant and the other incisor was its individual control. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM; Gibco Brl, Gaithersburg, MD, USA) supplemented with 15% foetal bovine serum (FBS; Gibco Brl, Gaithersburg, MD, USA). Exogenous Hsp 60 was added into the medium at 1  $\mu\text{g}\cdot\text{mL}^{-1}$  (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 min under healthy conditions.<sup>19,36</sup> The applied time period was important to follow the possible morphological changes of tooth germ. The culture medium was changed on the third day of culturing. The medium of treated explants was supplied with Hsp 60 (1  $\mu\text{g}\cdot\text{mL}^{-1}$ ). The experiment was concluded on the fifth day. The data of the *in vitro* cultures were based on four individual experiments. To determine morphological alternation of tooth germ, the angle between the labial and lingual root of the tooth germ at the level of the EK was measured in 5 days old tissue cultured samples in treated groups and in control groups ( $n = 7$ ). We used *in vitro* samples to avoid the unwanted side effects of dehydration during histology staining.

### Cell cycle analysis

A 10  $\mu\text{L}\cdot\text{mL}^{-1}$  5-bromo-2-deoxyuridine (BrdU) labelling reagent (Life Technologies, Carlsbad, CA, USA) was added to the culture medium for 2 h prior to fixation. The *in vitro* culture samples were fixed immediately in Sainte-Marie's fixative, dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections were cut in the

sagittal plane at 5–7  $\mu\text{m}$  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).

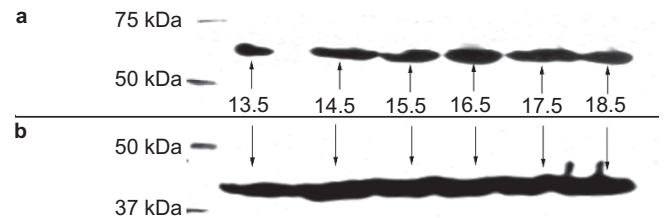
### Data analysis and image capturing

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, San Jose, CA, USA). Data for morphological analysis were measured with ImageJ 1.46 (National Institutes of Health, Bethesda, MD, USA). Statistical analysis was based on seven control samples and seven treated samples from four independent experiments. Significance of numerical data was verified by the Mann–Whitney *U*-test.

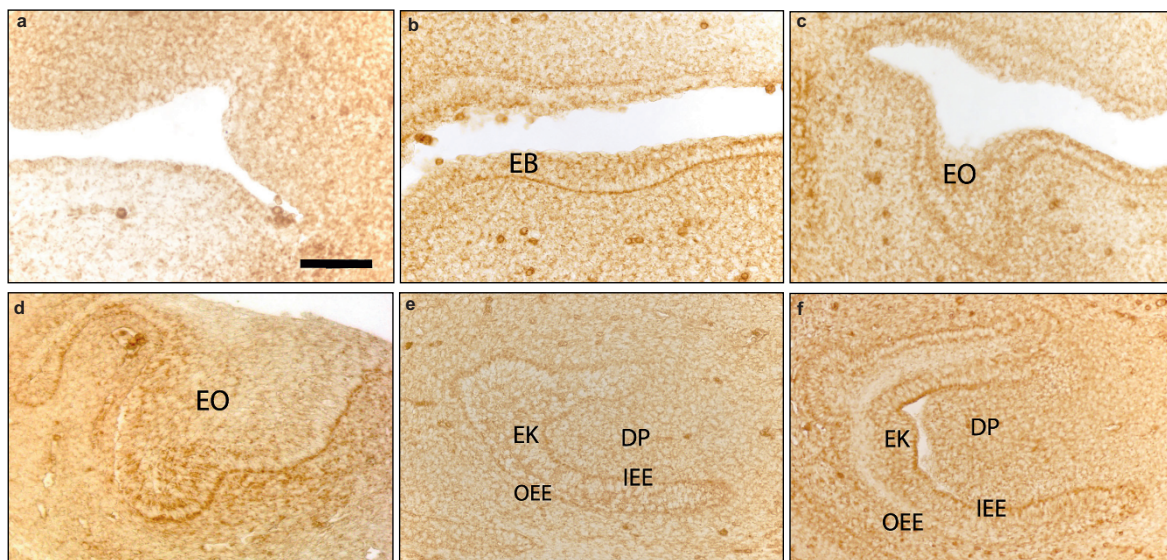
## RESULTS

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

We used Western blots to detect Hsp 60 protein in the tooth germ 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 (IHC) 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 (Figures 2 and 3).



**Figure 1** Results of Western blot analysis. (a) The presence of Hsp 60 in the tooth germ from E13.5 to E18.5; (b) the loading control (actin).



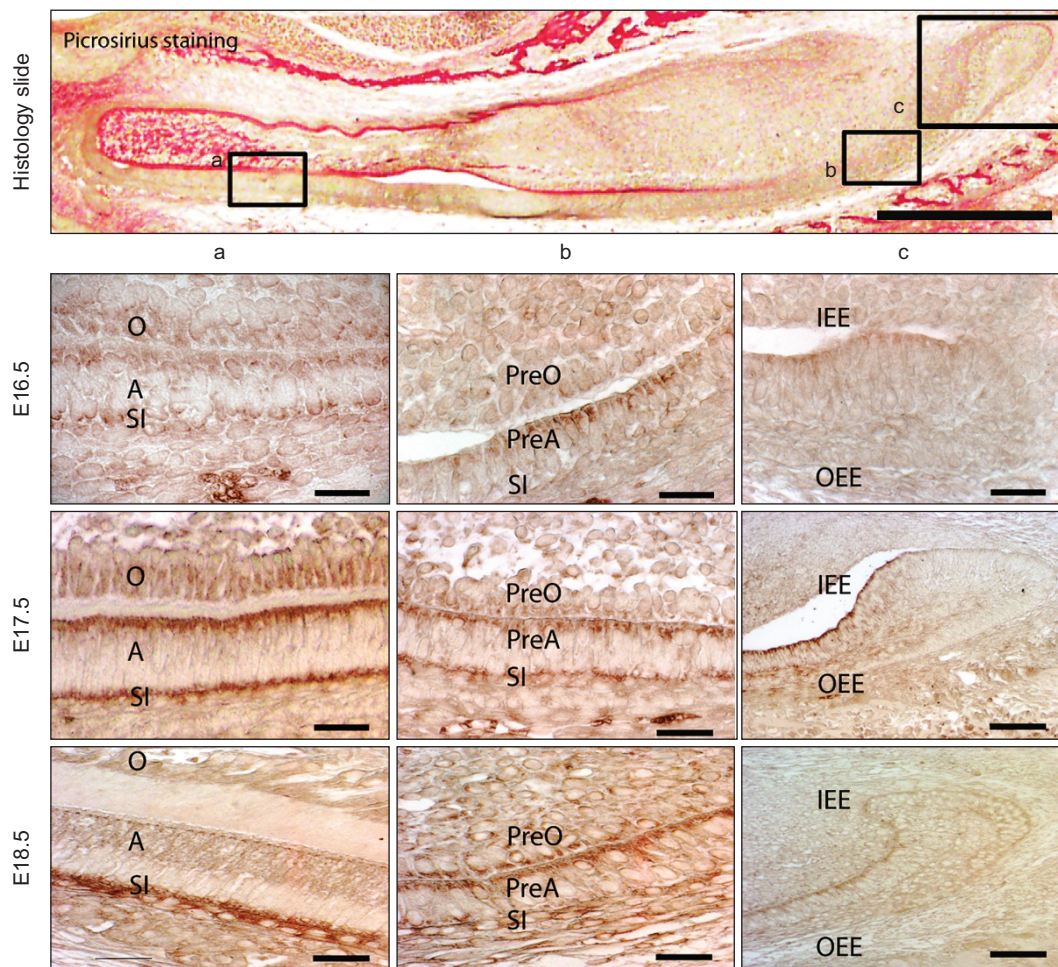
**Figure 2** The expression pattern of Hsp 60 in early stages of lower incisor development in mice. (a, b) Hsp 60 is present in the oral epithelium and EB in the initial stage of the tooth development. (c–f) From E12.5 to E15.5, Hsp 60 protein is present in the structures of the developing enamel organ (EO). From E14.5, the IEE, OEE and EK of the enamel organ were positive for Hsp 60. The cells of DP show weak Hsp 60 expression. a, E10.5; b, E11.5; c, E12.5; d, E13.5; e, E14.5; f, E15.5. Scale bar: a–d, 50  $\mu\text{m}$ ; e and f, 100  $\mu\text{m}$ . DP, dental papilla; EB, epithelialband; band; EK, enamel knot; EO, enamel organ; IEE, inner enamel epithelium; OEE, outer enamel epithelium.

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 (Figure 2b). During the bud and cap stage (E13.5–E15.5; Figure 2c–2f), the IEE and OEE and EK of the enamel showed intense Hsp 60 immunoreactive signals. In contrast, the DP and DF were weakly labelled (Figure 2c–2f), 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 IEE, OEE, preameloblasts (PreA), A, and SI (E16.5–E18.5; Figure 3). Moreover, homogenous DAB precipitations were found in the SI, which may indicate extracellular Hsp 60. The immunoreactivity increased in the cytoplasm of the preodontoblast (PreO) and odontoblast (O) cells at E16.5. A weak signal could be detected in the DF as well as in the surrounding mesenchymal tissue.

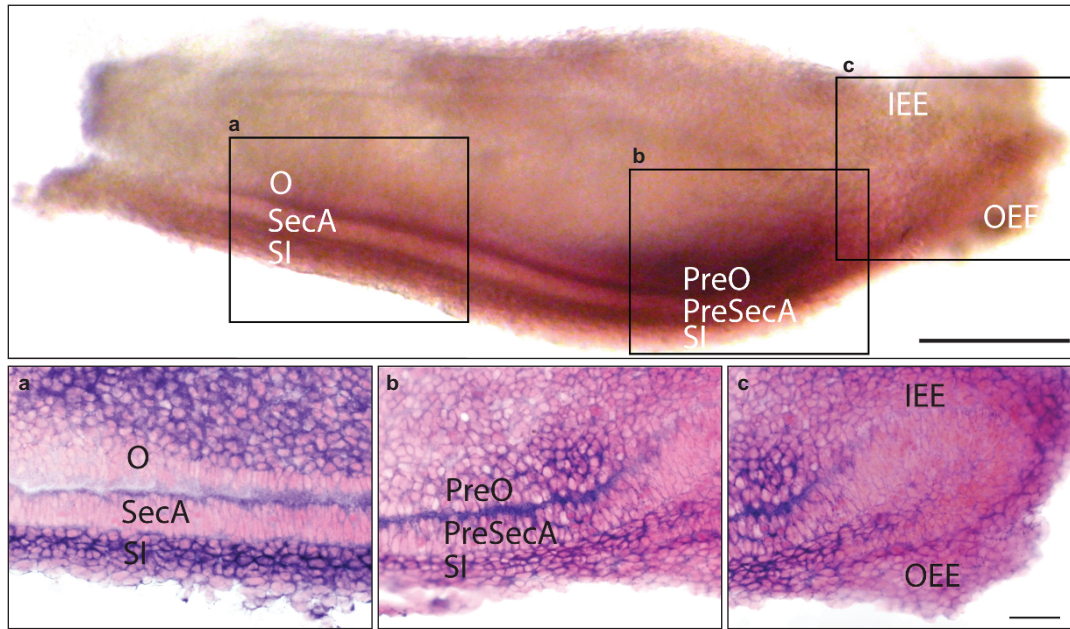
To enhance the result of IHC and to investigate the cellular origin of the extracellular Hsp 60 present in the SI at E16.5, we performed *in situ* hybridization. The majority of the Hsp 60 mRNA was localized to the labial side of the tooth germ, confirming the immunohistochemical results (Figure 4). A strong signal was observed in the cells of the OEE, IEE, PreA, A, SI, 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 germ

To further investigate the possible effects of extracellular Hsp 60 on tooth development, *ex vivo* organotypic tooth germ was 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 germ (Figure 5a and 5b). Altered morphology in treated cultures was first observed at the third DIV. The apical part of the treated tooth germ became blunted in shape with a clearly visible EK, whereas the proximal part of the tooth germ did not show any visual difference (Figure 5c and 5d). More profound morphological changes between the treated and control cultures were observed on the fifth day of culturing. The distal parts were sharp in the control explants and blunt in the treated explants. Similar to the earlier time points, the proximal part of the tooth germ showed no visual differences (Figure 5e and 5f). We performed picosirius histochemical staining to identify the morphology of the explants. Sections were oriented parallel to the longitudinal axis of the tooth germ. Distal parts of the treated tooth germ were blunt in comparison to the control samples (Figure 5g and 5h), confirming the observed macroscopic morphology described above with one important note: the altered side had a clear border and living



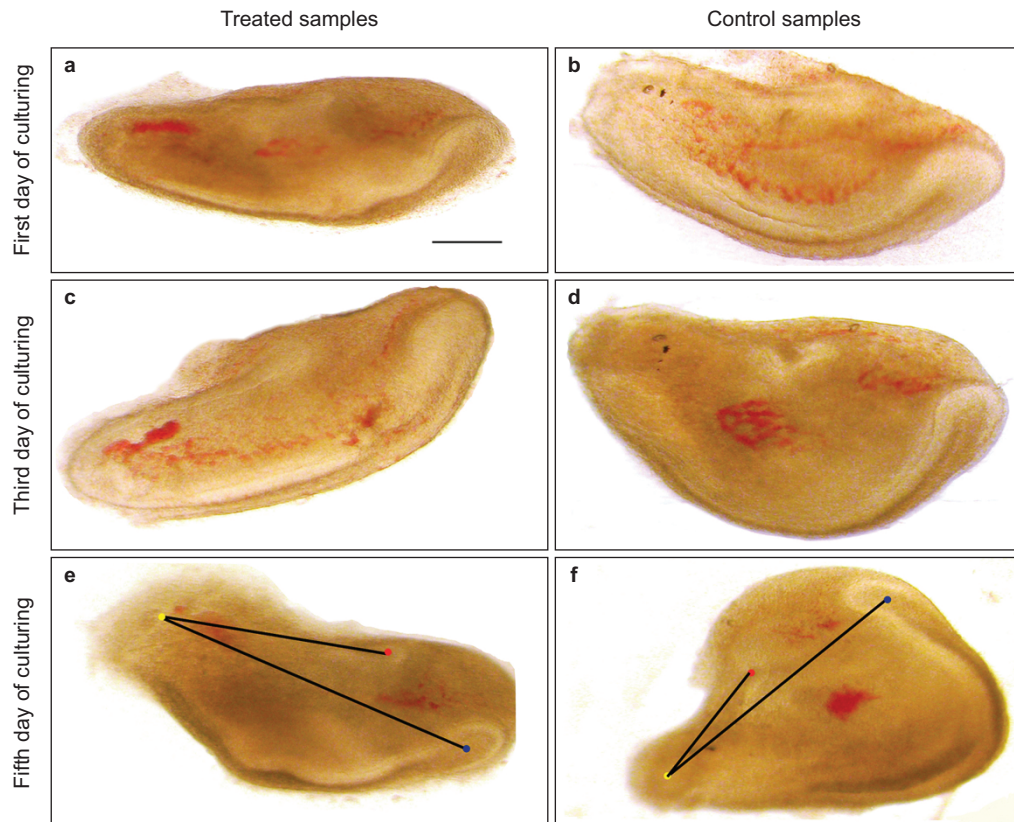
**Figure 3** The expression pattern of Hsp 60 during the bell stage of lower incisor development. A histology slide was stained by picosirius F3B to help recognize the position of inserts of **a**, **b** and **c** columns from different stages (E16.5–E18.5). (**a**) The apical part of the enamel organ; (**b**) the proximal part of the enamel organ; (**c**) the labial root sheet. PreA, A, SI, IEE and OEE abundantly contain Hsp 60 signals. PreO and O also show intensive immunoreactions. From E17.5, the SI shows intensive Hsp 60 immunolabelling. Scale bar: 500 µm; **a** and **b** inserts, 20 µm; **c** inserts, 100 µm. A, ameloblasts; IEE, enamel epithelium; O, odontoblasts; OEE, outer enamel epithelium; PreA, preameloblasts; PreO, preodontoblasts; SI, stratum intermedium.

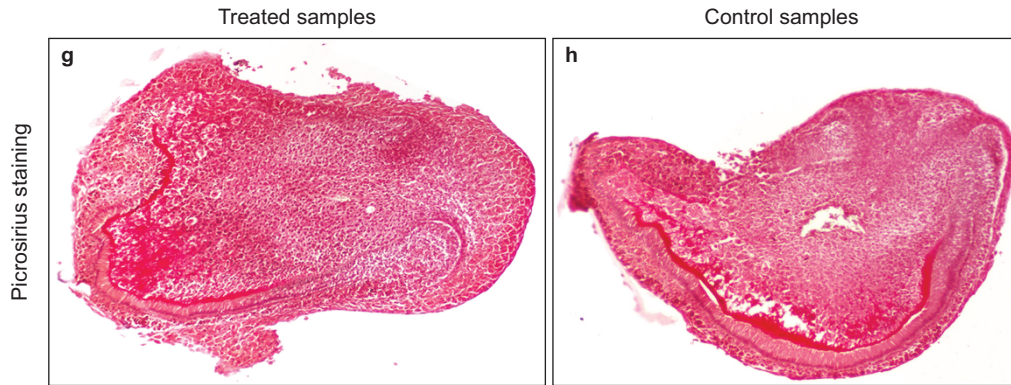


**Figure 4** Results of *in situ* hybridization on 16.5-day-old tooth germ. To verify the results of immunohistochemistry, we performed *in situ* hybridization, which confirmed the enhanced expression of Hsp 60 in the enamel organ and in odontoblasts. (a) Apical part of the enamel organ at a higher magnification; (b) proximal part; (c) labial root from the whole mount sample. The probe positively labelled the PreA, A, PreO, O, SI and OEE of the tooth germ. The IEE also shows a signal that is not very intense. Scale bar: 100  $\mu$ m; a–c inserts, 20  $\mu$ m. A, ameloblasts; IEE, enamel epithelium; O, odontoblasts; OEE, outer enamel epithelium; PreA, preameloblasts; PreO, preodontoblasts; SI, stratum intermedium.

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 5e and

5f) were closed to a 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 histological observation (Figure 5g and 5h).



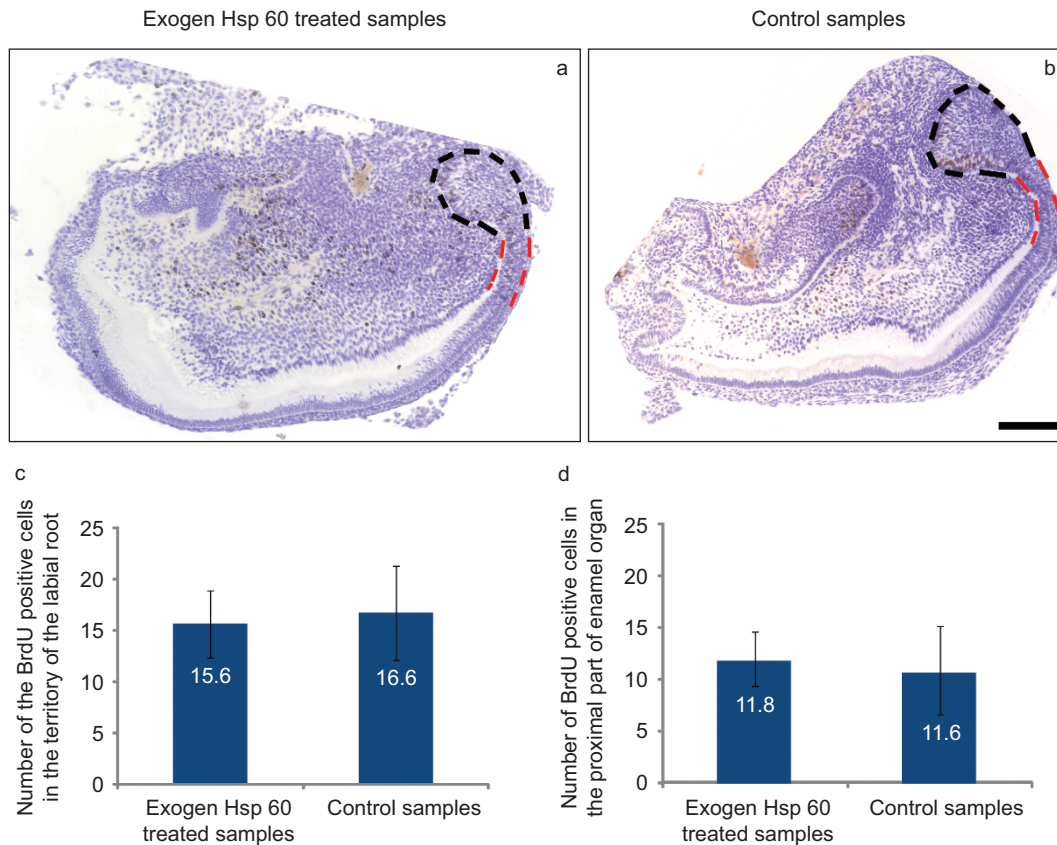


**Figure 5 Exogenous Hsp 60 causes abnormal morphology of the *in vivo* cultured E16.5 incisor.** (a,b) At the end of the first day in culture, no visual difference can be observed between the treated and control tooth germ. (c,d) The first morphological sign appears on the third day of *in vitro* culture. The distal part of the treated samples starts to show blunted apical parts while the apical part of the control sample shows normal morphology. (e,f) On the fifth culture day, clear morphological differences develop between our samples. The treated tooth germs have blunted distal parts while the control samples have sharp distal parts. A red point indicates the centre of the lingual loop, a blue point indicates centre of the labial loop and a yellow point indicates the enamel knot. The degree of enclosure among these points in the case of the treated samples is significantly higher ( $P > 0.05$ ). (g,h) After the histochemical staining, more obvious differences develop between the two groups. The treated tooth germs have abnormal blunted distal parts while the control samples have normal morphology. The intense red colour indicates collagen in the predentin. Scale bar: a–f, 100  $\mu$ m; g and h, 50  $\mu$ m.

### Cell cycle analysis

We used the BrdU incorporation test to detect the dividing cells of the tooth germ. 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 the treated and the control groups. Although the distribution of BrdU positive cells seemed slightly different in the two experimental groups, we did not find



**Figure 6 Effect of Hsp 60 on the proliferation of dental cells.** Proliferating cells incorporating 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 and b, c) and in the enamel organ (investigated territory bounded by red dashed lines on a and b, d). 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  $\mu$ m. BrdU, 5-bromo-2-deoxyuridine.

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 Hsp are essential components of the organisms, although they had diverse functions and various expression patterns during tooth development. During odontogenesis, the expression patterns and possible roles of Hsp 25, Hsp 27, Hsp 86, Hsc73 and Hsj2 were already described.<sup>37–41</sup> Among these, the Hsp 25 has been studied the most in rat and mice incisors.<sup>37–38</sup> The dental pulp, PreO and A were transiently positive for Hsp 25, while the odontoblasts were shown to have continuous Hsp 25 expression.<sup>38</sup> The Hsp 27 was detected in the dental epithelium of the bell stage, and Hsp 27 might be related to the morphological development of the tooth.<sup>41</sup> The distribution pattern of Hsp 86, Hsj2 and Hsc73 was similar to each other in the enamel organ. The IEE and primary EKs showed strong signals during the bell stage.<sup>40</sup> According to our findings about Hsp 60, we found a similar expression pattern with Hsp 25 and Hsp 27. More intense immunoreactions 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, a whole mount *in situ* hybridisation against mRNA of Hsp 60.

The Hsp 60 has several intracellular functions under healthy conditions.<sup>24–27</sup> 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 TLR4 receptor signalling. Exogenous Hsp 60 can be taken up by TLR4 receptors, which can activate the NF- $\kappa$ B signalling pathway.<sup>42–43</sup> In our study, excess Hsp 60 caused an abnormal morphology (Figure 5) of the distal part of the tooth germ. In these experiments, with the applied high level of Hsp 60 during the *in vitro* culturing model, we wanted to mimic the increased levels of Hsp 60. The increased levels of Hsp 60 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 with Western blots showed the presence of TLR4 receptor in the tooth germ, which strengthens our hypothesis for downstream action of Hsp 60 through 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.<sup>44–45</sup>

The ectodysplasin/TNF signalling pathway may also take part in the effect of Hsp 60. This pathway has a major role in the morphological development of ectodermal appendices, and the downstream portion of the signalling pathway includes the IKK complex.<sup>46</sup> The IKK complex consists of three subunits: IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ <sup>46</sup> and plays an NF- $\kappa$ B-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.<sup>47–48</sup> Recent *in vitro* culture experiments support earlier findings in which free cytosolic Hsp 60 can attach to the IKK complex, and the absence of IKK $\alpha$  can cause abnormal tooth phenotypes.<sup>19,49–50</sup> Similar morphology was observed for IKK $\alpha$  knockout mice between the treated incisor tooth germ and the lower incisors.<sup>47</sup> However, IKK modifies the degradation of I $\kappa$ B, resulting in normal tooth morphology in the I $\kappa$ B knockout mice. This indicates that the blunted distal part of the treated tooth

germ in our experiments probably is not the result of altered expression of NF- $\kappa$ B target genes.<sup>47</sup> This background supports that the Hsp 60 may attach to IKK $\alpha$  and that this connection may inhibit the function of IKK $\alpha$  in developmental processes.<sup>19</sup>

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 IEE.<sup>51–52</sup> According to the literature, Hsp 60 may increase the proliferation rate of the epithelium-derived cells.<sup>53</sup> However, this mitogenic effect of Hsp 60 could not be confirmed in dental cells. We also investigated the apical part of the tooth germ, which is responsible for forming the shape of the tooth.<sup>54</sup> The EK is a transient structure that is responsible for the final morphology of the tooth and does not contain proliferating cells in the bell stage.<sup>55–56</sup> This was not altered by exogenous Hsp 60. Our results suggest 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 of the expression pattern of Hsp 60 mRNA and protein during tooth development. According to our results, this protein can play a role in the morphological development of tooth germ. Although the exact mechanism by which Hsp 60 influences morphogenesis of tooth germ has not been clarified, our results suggest the involvement of the IKK complex. This study supported the roles of Hsp during developmental processes. The elevated amount of Hsp 60 may be the result of pathological processes.

## ACKNOWLEDGEMENTS

A University of Debrecen (RH/885/2013) PhD grant made it possible to perform our experiments. This research was also supported by the European Union and the State of Hungary and co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program'. Szabolcs Felszeghy was supported by a János Bolyai fellowship from the Hungarian Academy of Science. The authors thank Prof. László Módis for critical reading of the manuscript and his valuable comments. We acknowledge the editing services of American Journal Experts.

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