ARCHIVES OF ORAL BIOLOGY 53 (2008) 791–799

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/arob

Expression of MMP-13 (collagenase-3) in long-term cultures of human dental pulp cells

Lokesh Suri [a,*], *Petros D. Damoulis* [b], *Trang Le* [c], *Eleni Gagari* [d]

[a] *Department of Orthodontics, DHS-2, Tufts University, School of Dental Medicine, One Kneeland Street, Boston, MA 02111, USA*
[b] *Department of Periodontology, Tufts University, School of Dental Medicine, One Kneeland Street, Boston, MA 02111, USA*
[c] *Tufts University, School of Dental Medicine, One Kneeland Street, Boston, MA 02111, USA*
[d] *Department of Oral and Maxillofacial Pathology, Tufts University, School of Dental Medicine, One Kneeland Street, Boston, MA 02111, USA*

ARTICLE INFO

Article history:
Accepted 2 February 2008

Keywords:
MMP-13
Collagenase-3
Dental pulp
Stem cells
Mineralization

ABSTRACT

Matrix metalloproteinase-13 (MMP-13 or collagenase-3) is a member of the family of matrix metalloproteinases (MMPs) produced in high amounts by cells with mineralising potential. Human dental pulp has been shown to express high levels of MMP-13 RNA.

Objective: Since human dental pulp derived cells (HDPC) are known to possess osteoprogenitor properties, we investigated the pattern of expression of MMP-13 in long-term cultures of those cells under conditions that support mineralisation *in vitro*.

Design: Impacted teeth or teeth extracted for orthodontic purposes were used to obtain dental pulp explants and HDPC were cultured for approximately 5 weeks. Pro- and active MMP-13 levels were determined in the cell culture supernatants by means of enzyme-linked immunosorbent assay (ELISA). Cell growth was evaluated through DNA content and osteogenic differentiation was assessed by alkaline phosphatase (ALP) activity and Alizarin Red staining.

Results: Mineralising cultures of HDPC produced significantly higher levels of pro-MMP-13 compared to control cultures. Both pro- and active MMP-13 levels displayed a characteristic peak that was found to coincide with the peak in alkaline phosphatase activity and the onset of mineralisation. Once mineralisation was firmly established, MMP-13 expression was significantly reduced.

Conclusions: Evidence from this study suggests a role for MMP-13 in the transition of human dental pulp cells to a mature mineralising phenotype and points to MMP-13 as a possible marker in HDPC differentiation.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of extracellular matrix (ECM)-degrading enzymes that traditionally are known to play a role during matrix formation and turnover.[1,2] They are synthesised and secreted as transmembrane proenzymes that are processed to an active form by the removal of an amino-terminal peptide. Recently, their role has also been highlighted in the regulation of cellular differentiation.[3,4] A variety of MMPs have been associated with cellular differentiation in different tissues such as bone[4], adipose tissue[5], ovarian tissue[6], as well as in spermatogenesis[7] and in monocyte-macrophage differentiation[8]. More specifically, MMP-13 (also called collagenase-3) expression has been

* *Corresponding author*. Tel.: +1 617 636 2489; fax: +1 617 636 2740.
E-mail address: lokesh.suri@tufts.edu (L. Suri).
0003–9969/$ – see front matter
doi:10.1016/j.archoralbio.2008.02.010

closely associated with chondrocyte and osteoblast differentiation and the formation of a mineralised extracellular matrix.[9–13] MMP-13 expression has been reported to increase in hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development.[14] Tuckermann et al. investigated the temporal and spatial expression of MMP-13 *in vivo* at sites of endochondral bone formation by *in situ* hybridisation and concluded that MMP-13 could be used as a marker for osteoblastic differentiation.[9] It has also been reported that transcriptional regulation of the MMP-13 gene depends on the synergistic action of the transcription factors AP-1 and RUNX2 that also regulate osteoblast differentiation.[15,16]

The human dental pulp cell (HDPC) colonies that arise from pulp explants have been variedly described as fibroblasts[17,18], fibroblasts with osteogenic potential[19], myofibroblasts[20], odontoblasts[21], pericytes[22] and stem cells.[23] One common feature of HDPs is their ability to lay down matrix and form mineralised nodules.[19,21,24] Recent studies have further characterised HDPC as mesenchymal stem cell populations and compared them to bone marrow stromal stem cells (BMSCs).[23,25] HDPC and BMSCs have been found to be comparable with respect to their expression of a variety of markers including osteocalcin, osteonectin, alkaline phosphatase (ALP), collagen, peroxisome proliferator-activator receptor γ (PPAR γ), MyoD, CD14 and α-smooth muscle actin. Furthermore, HDPC clearly possess osteoprogenitor characteristics, as they can highly express osteoblastic lineage markers such as collagen, RUNX2, alkaline phosphatase, and osteocalcin.[23,25–27]

Although the presence of a number of different MMPs and, in particular, MMP-13, has been reported in mature odontoblasts as well as in healthy and inflamed pulp tissue,[28,29] the expression pattern and role of these enzymes in dental pulp cells is unclear. In this study, we propose to demonstrate that human dental pulp cells produce MMP-13 in high levels and we aim to investigate the association of MMP-13 expression with the biologic process of mineralisation in HDPC cultures.

2. Materials and methods

The collection and use of human extracted teeth as a source for HDPC for this project was approved by the Tufts-New England Medical Center Institutional Review Board. Impacted teeth or teeth removed for orthodontic purposes from the Oral and Maxillofacial Surgery Clinic were used to obtain pulp explants. On average, three teeth (premolars or molars) were obtained for the generation of each primary culture. Only teeth free of caries, restorations or any associated pathology were taken for the generation of HDPC cultures.

2.1. *Human dental pulp tissue culture*

All chemicals were purchased from Sigma (St. Louis, MO, USA) and all tissue culture reagents from BioWhittaker (Walkersville, MD, USA), except when stated otherwise. Each tooth was split open with a chisel and mallet and the pulp removed with a sterile spoon excavator and tweezers in a sterile tissue culture hood environment. The pulp was subsequently minced using sterile scissors and aseptically transferred to the culture medium. The pulp fragments were cultured in Eagle's Modified Minimal Essential Medium alpha supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA), 2 mM L-glutamine, 100 IU/mL Penicillin, and 100 μg/mL streptomycin (αMEM) in plastic tissue culture flasks (T-75, Corning Incorporated, Corning, NY, USA). The explants were incubated at 37 °C in a incubator at 5% CO_2 and monitored for cell attachment and growth. Medium was changed on days 7 and 10 and cells were then passaged on day 14 (P-1). Five to seven days later, an adequate number of cells were obtained for the planned experiments. All experiments were performed at P-2. This cell culture model has been well established in our laboratory[30] and has been extensively characterised through flow cytometry and gene microarray analysis. Invariably, these cells express high levels of CD44 and CD146, consistent with their mesenchymal origin and stem cell like properties (data not shown).

HDPC were seeded in 6-well plates (Corning Incorporated, Corning, NY, USA) at a concentration of 5.5×10^2 cells/cm^2. Media was changed on day 2 post-seeding and at 3-day intervals thereafter, for the duration of the experiment. Two parallel experiments were performed; Experiment I in complete medium and Experiment II in serum-free conditions. Serum-free conditions were employed to allow for the measurement of active-MMP-13. Experiment I: upon confluence (day 8) the experimental plates were divided into two groups. In the control group no additives were used, whereas in the mineralising group αMEM was supplemented with 50 μg/mL ascorbic acid (Invitrogen, Grand Island, NY, USA) and 50 mM β-glycerol phosphate at every media change. Supernatants and cell lysates were collected on days 8, 11, 14, 17, 20, 26, 32 and 38. The supernatants were used to assay for the presence of the pro-form of MMP-13 (pro-MMP-13) by enzyme-linked immunosorbent assay (ELISA). The cell lysates were used for DNA quantification as well as measurement of alkaline phosphatase activity. Determination of matrix mineralisation was performed with the Alizarin Red S dye on days 26, 32 and 38 post-seeding.

In the parallel experiment (Experiment II), cells were cultured under mineralising conditions as previously described, until day 26. At that point, an additional serum-free group was established, where FBS was substituted for 0.1% bovine serum albumin (BSA) and insulin-transferrin-selenium (ITS) until day 32. These time points were selected to correspond with initiation of mineralisation. Medium was changed on day 29 and samples were collected on days 26, 27, 28, 29 and 32. Supernatants were used for the presence of pro-MMP-13 and active form of MMP-13 (active-MMP-13) by ELISA and cell lysates for DNA quantification. Alizarin Red S dye staining was also performed at each time point for the assessment of matrix mineralisation.

2.2. *Sample collection*

Culture supernatants were collected at each time point, aliquoted and frozen at −80 °C until the time of analysis. Once the supernatant was collected, the cell layer was washed twice with ice cold 50 mM tris-buffered saline, pH 7.5 (TBS),

lysed in 1% Triton-X (Fisher Scientific, Suwanee, GA, USA) in TBS and the lysate was sonicated to disrupt the cell membranes. Any triton insoluble fragments were removed by centrifugation. The lysates were also aliquoted and frozen at −80 °C until the time of analysis.

2.3. DNA quantification

Double stranded DNA in the cell lysates was measured fluorometrically by mixing 100 μL of diluted cell lysate with 100 μL of a nucleic acid stain solution (PicoGreen, Molecular Probes, Eugene, OR, USA) in an opaque 96-well plate (Corning Incorporated, Corning, NY, USA). A standard curve of salmon testis DNA was also prepared in the same way. Fluorescence was measured with a microplate fluorometer (MFX, Dynex Technologies, Chantilly, VA, USA) using 480 nm excitation and 520 nm emission wavelengths. Values were normalised to lysis volume and expressed as μg DNA per well.

2.4. Lactate dehydrogenase (LDH) release assay

LDH is an abundant intracellular enzyme, and its release into tissue culture supernatants is a standard indicator of lytic cell death.[31] LDH in cell-free supernatants diluted fivefold with PBS was quantified colorimetrically with a coupled enzymatic assay which results in the conversion of a tetrazolium salt (INT) to a red formazan product, using a kit (Promega, Madison, WI). Absorbance (A) was measured at 490 nm and results are expressed as percentage LDH enrichment = $(A_{experimental}/A_{control}) \times 100$. Control represents maximum LDH release after cells are lysed with a detergent.

2.5. Determination of alkaline phosphatase activity

Alkaline phosphatase activity was measured in the cell lysates through the conversion of p-nitrophenyl phosphate (pNPP) to p-nitrophenyl (pNP), which can be measured spectrophotometrically.[32,33] 80 μL of the cell lysate were mixed with 120 μL of 10 μM pNPP in 2-amino-2-methyl-1-propanol buffer (Sigma Diagnostics, St. Louis, MO, USA). The reaction was stopped after 5–15 min (depending on colour intensity) with 100 μL of 0.2 N NaOH. Within this time frame, the intensity of colour formed is proportional to phosphatase activity. Values were plotted against a pNP standard curve, normalised to lysis volume and expressed as nmoles pNP/min/μg DNA.

2.6. Pro-MMP-13 quantification

Pro-MMP-13 was quantified in the supernatant using a commercially available enzyme-linked immunosorbent assay kit (R&D systems, Minneapolis, MN, USA). Each sample was assayed in duplicate. The samples and standards were quantified with a microplate reader (MRX Microplate reader, Dynex Technologies, Chantilly, VA, USA) capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm. Pro-MMP-13 quantity in samples was calculated by linear regression analysis. The amount of pro-MMP-13 was expressed as picograms/well.

2.7. Active-MMP-13 quantification

Supernatants from serum-free cultures were used for quantification of active form of MMP-13 by a commercially available ELISA (R&D Systems, Minneapolis, MN). Each sample was assayed in duplicate and the samples and standards were quantified fluorometrically with a fluorescence plate reader (MFX Microtiter® Plate Fluorometer, Dynex technologies, Chantilly, VA, USA) with excitation wavelength set to 320 nm and emission wavelength set to 405 nm. The amount of active MMP-13 was expressed as picograms/well.

2.8. Determination of matrix mineralisation (Alizarin Red S staining)

For the later time points (days 26, 32 and 38 for Experiment I and days 26, 27, 28, 29, 32 for Experiment II), matrix mineralisation was determined with the Alizarin Red S dye.[34] Alizarin red dye chelates calcium and the resulting complex has a bright red colour, the intensity of which is proportional to the amount of calcium present. Cells were fixed with 70% ethanol at 4 °C for 2 h and stained with 1% Alizarin Red S solution, pH 4.2, for 15 min. Excess dye was removed with repeated water washes followed by a 15 min wash with PBS, after which the plates were photographed. The wells were subsequently destained by incubation with 10% cetylpyridinium chloride in 10 mM sodium phosphate solution for 15 min. 200 μL of the resulting purple solution were transferred to a 96-well plate and the absorbance was measured at 562 nm.

2.9. Data analysis

Values are presented as mean and standard error of mean from six independent experimental samples. Statistical significance was evaluated using the two-tailed Student's t-test to compare values between two groups at each time point, and ANOVA to compare differences between time points within the same group.

3. Results

3.1. HDPC in long-term culture differentiate and produce a mineralised matrix

Differentiation was promoted by the addition of ascorbic acid and β-glycerol phosphate, which assist in the development of a mineralised extracellular matrix. Since addition of these agents started at confluence, we did not observe dramatic differences in DNA content between the control and mineralising groups throughout the experiment. However, the mineralising group consistently showed higher DNA values, probably reflecting greater cell numbers (Fig. 1). Both groups presented a decline in DNA content after day 26, which was more pronounced in the mineralising cells. LDH release into culture medium increased over time in both groups but was significantly higher in the mineralising cultures at all time points (Fig. 2). A peak in LDH level was observed in the mineralising cultures at day 32. Alkaline phosphatase activity,

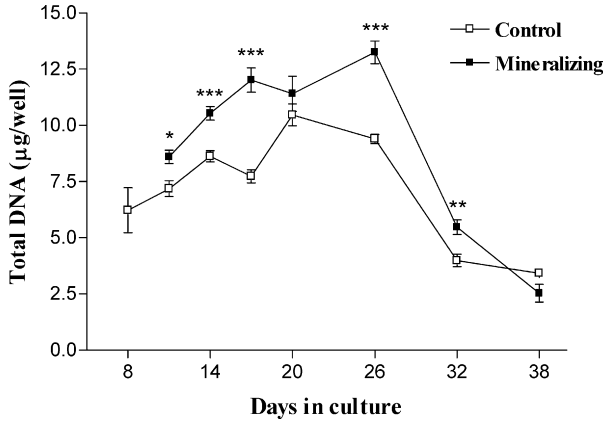

Fig. 1 – DNA content of HDPC in control and mineralising groups. Addition of mineralising medium started on day 8 (confluence). Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

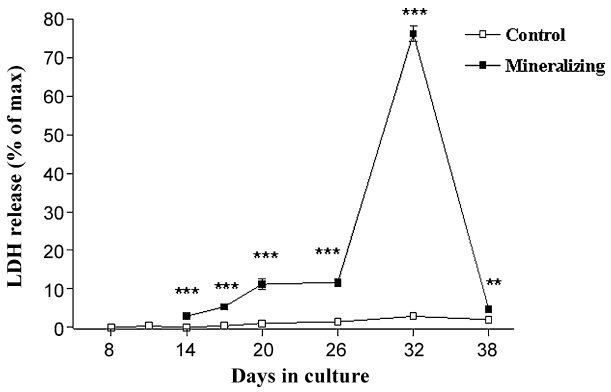

Fig. 2 – LDH release assay in control and mineralising HDPC cultures. Addition of mineralising medium started on day 8 (confluence). Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

a marker of osteoblastic differentiation, was also monitored throughout the culture period (Fig. 3). HDPC expressed low levels of ALP activity under non-differentiating conditions with a moderate increase as the culture aged. When confluent cultures were stimulated with β-glycerol phosphate and ascorbic acid however, ALP activity displayed an initial peak followed by a decline, a pattern often observed in cultures of cells with osteogenic potential (Fig. 3).

Calcium incorporation, reflecting matrix mineralisation, was measured by Alizarin Red S staining (Fig. 4a and b). Cultures maintained in αMEM did not mineralise, whereas the addition of ascorbic acid and β-glycerol phosphate resulted in the detection of visible calcium deposition by day 32, which was consolidated by day 38. ALP activity also peaked on day 32, coinciding with the onset of mineralisation. The visual

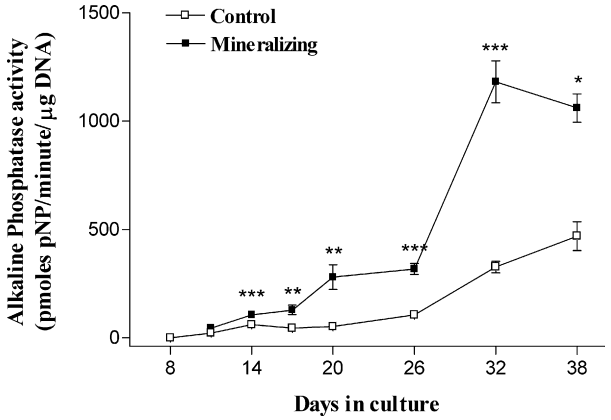

Fig. 3 – Alkaline phosphatase (ALP) activity in control and mineralising HDPC cultures. Addition of mineralising medium started on day 8 (confluence). ALP activity was determined in cell homogenates and value of ALP expression is normalised to DNA content. Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

assessment of mineralisation was confirmed by measuring the intensity of the stain colorimetrically (Fig. 4b). Interestingly, a small difference in stain intensity was detected as early as day 26, potentially representing a small subpopulation of cells, which mineralised earlier. Minor variations in the onset and degree of mineralisation in separate experiments where different primary cultures were used were encountered, however the overall results were highly reproducible. Fig. 4 represents the typical mineralisation course in our HDPC culture model.

In Experiment II, HDPC were cultured under mineralising conditions as described previously, but a serum-free [FBS (−)] group was added on day 26. The growth curves of FBS (+) and FBS (−) groups from days 26 to 32 were essentially identical, indicating that serum deprivation did not alter the growth characteristics of these cells at that stage in culture. (Fig. 5) Calcium incorporation in the mineralising matrix was also similar, evaluated by Alizarin Red S staining (Fig. 6). The first evidence of mineralisation appeared on day 28 with a continuously increasing staining intensity on days 29 and 32. The visual assessment of mineralisation was confirmed colorimetrically and no statistically significant differences were detected between the FBS (+) and FBS (−) groups (data not shown).

3.2. *HDPC produce measurable levels of pro-MMP-13 under mineralising conditions*

Pro-MMP-13 was barely detectable in the cell culture supernatants from the control group, but was significantly upregulated in HDPC under mineralising conditions (Fig. 7). This difference was highly significant ($p < 0.001$) at each time point. Statistically significant differences were also found within the mineralising group, when sequential time points

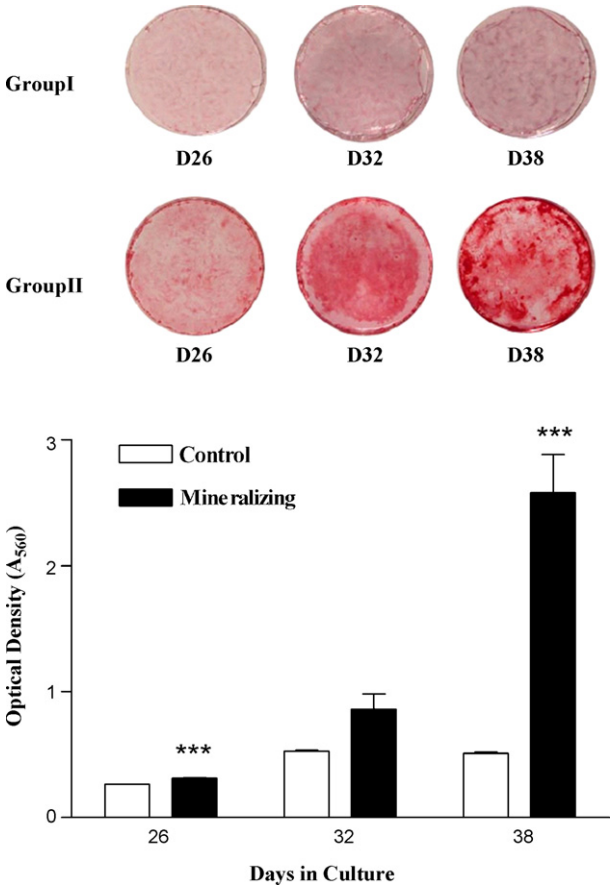

Fig. 4 – (a) Mineralisation assay: Alizarin Red S staining for detection of mineralisation in control and mineralising HDPC cultures. Representative wells from each group at days 26, 32 and 38 are shown. (b) Quantification of staining intensity represented as an average of optical density. Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

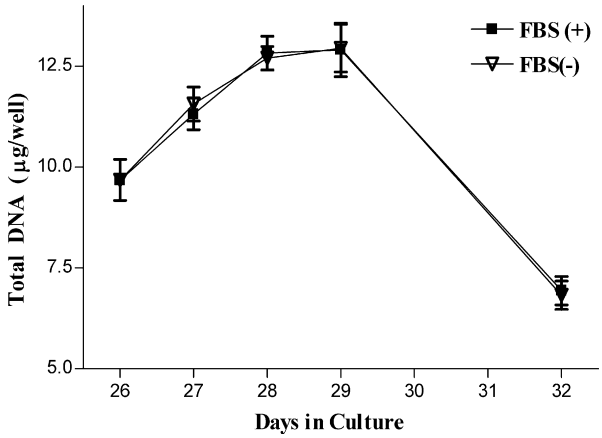

Fig. 5 – DNA content of HDPC under mineralising conditions using standard [FBS (+)] and serum-free [FBS (−)] medium. Serum deprivation did not alter the DNA content during the starvation period (days 26–32). Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

were compared. This indicated a significant increase in pro-MMP-13 secretion as the cells differentiated in culture. The most striking observation however was the fourfold increase of pro-MMP-13 on day 32 (Fig. 7). This peak coincided with the highest ALP activity and the onset of mineralisation. After that, pro-MMP-13 levels declined dramatically, reaching almost baseline values on day 38 ($p < 0.001$ compared to day 32).

3.3. *Onset of mineralisation coincides with increased MMP-13 activity in HDPC*

In Experiment II, a serum-free group was established to allow measurement of active MMP-13, since the presence of serum-derived protease inhibitors can mask enzymatic activity. The FBS (−) group showed a progressive increase in detectable amounts of active-MMP-13 from day 27 through day 29, mirroring an increase in pro-MMP-13 levels (Fig. 8a and b). In this experiment, the association between MMP-13 levels and mineralisation was more closely monitored. The first evidence of mineralisation was seen on day 28 for both FBS (+) and FBS (−) groups (Fig. 6), indicating that MMP-13 activity starts to increase at the onset of mineralisation and remains elevated for a period of time afterwards. Maximal ALP activity was also observed on day 28 (data not shown), confirming the results of Experiment I. The daily collection of samples in Experiment II also allowed normalisation of MMP-13 to DNA content. Normalised values of active MMP-13 showed a continuous increase with a faster rate of increase from days 28 to 29 (Fig. 8a). Normalised pro-MMP-13 levels also showed the same trend in both groups (Fig. 8b). The mean values for days 27, 28, 29 were 125.9/228.6/843.9 pg/μg DNA respectively for the FBS (+) group and 171.1/217.9/376.8 pg/μg DNA for the FBS (−) group. Maintaining the cells in serum deprivation condition beyond day 32 did not contribute further significant information.

4. Discussion

The human dental pulp, like other post-natal tissues, has been shown to have resident progenitor cells. These cells have been reported to have stem cell like properties and are capable of differentiation in several pathways both *in vitro* and *in vivo*.[23,25] Our experiments and those of other investigators have demonstrated that explants from the human dental pulp provide a cell population *in vitro* that primarily represents cells with mineralising potential. In the osteoblastic lineage, differentiation of an osteoprogenitor cell into a functional osteoblast requires the sequential expression of several genes[35] including MMP-13.[9] In this study we attempted to

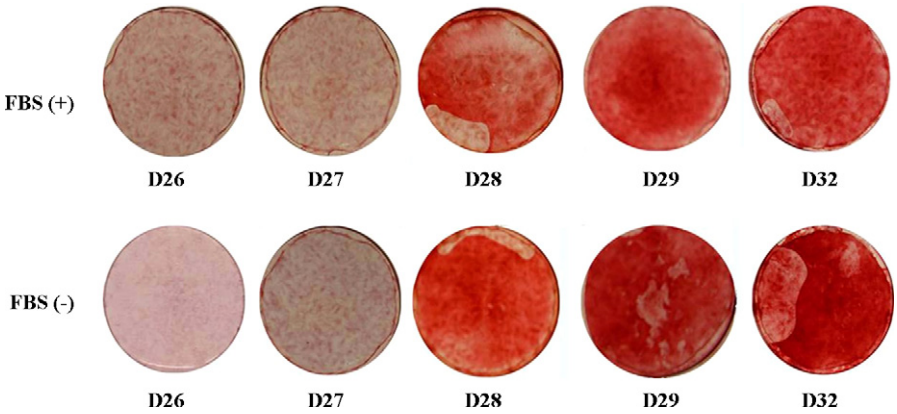

Fig. 6 – Mineralisation assay: Alizarin Red S staining in FBS (+) and FBS (−) HDPC cultures. Representative wells from each group at days 26, 27, 28, 29, and 32 are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

characterise the expression of MMP-13 and evaluate its functional relationship with mineralisation in HDP-derived progenitor cells.

We have established an HDP-derived cell culture model that is reproducible and consistent with what has been reported in the literature.[19,23–26] Phenotypically, HDPC in culture are similar to that of the well-established osteoprogenitors of bone marrow aspirates. Both cell types exhibit a fibroblast-like morphology, adhere to a plastic substratum, have the capacity to form clonogenic cell clusters and lay down a mineralised extracellular matrix.[25] By assaying DNA content, ALP activity and mineral presence we were able to follow the transition of these cells from a progenitor to a mineralising phenotype. Differentiation associated apoptosis and cell death was monitored by the LDH release and found to peak at mineralisation. The progression towards a mineralising phenotype involves the regulation of expression for a variety of genes. However, there is extensive information in the literature on changes of expression of those genes in osteoblastic, chondrocytic as well as in HDP-derived cells. Data from gene array analysis performed in our laboratory has shown that HDPC in long-term culture consistently express high levels of extracellular matrix molecules and the development of a mineralising phenotype is associated with increased expression of bone morphogenetic protein (BMP)-2 and associated signaling pathways. Furthermore, we have identified the mineral formed as hydoxyapatite but its exact structure is still unclear (data not shown). Therefore, we considered that ALP activity and mineralisation were adequate markers to monitor osteogenic differentiation in this cell culture model.

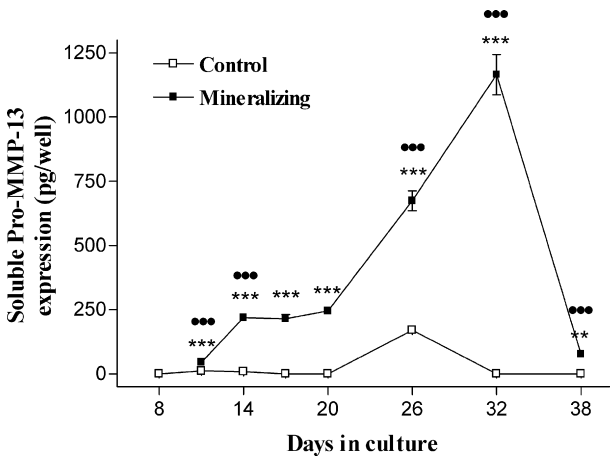

Fig. 7 – Expression of pro-MMP-13 in HDPC under non-mineralising and mineralising conditions. Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups and bullets indicate statistical significance within the mineralising group between sequential time points: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; $^{\bullet\bullet\bullet}p < 0.001$, $^{\bullet\bullet}p < 0.01$, $^{\bullet}p < 0.05$.

Consistent with the differentiation profile of osteoprogenitors from bone marrow,[19,21,25] ALP activity was significantly higher in mineralising cultures at all time points assessed. (Fig. 2) Furthermore, ALP activity under mineralising conditions increased significantly during the 5th week of culture reaching a peak on day 32, which coincided with a dramatic decrease in DNA content (Figs. 1 and 2). A similar pattern is also observed during osteoblast differentiation *in vitro*.[36] Presence of ascorbic acid and β-glycerol phosphate was essential for the HDPC to form a mineralised matrix, as has been well established in mineralisation studies with osteoblasts, chondroblasts and osteoprogenitors of bone marrow origin.[12,36–38] Additional differentiation factors such as dexamethasone and BMP-2 were not required for mineralisation. Mineralisation was observed as early as 3.5 weeks (Fig. 3), whereas other investigators report that a 5–6-week period is required.[25] This variability in the time of onset of mineralisation may be attributed to the heterogeneity among primary culture populations as well as the difference in culture conditions. It appears that the age of the donor and developmental stage of the tooth from which HDP explants were obtained may also influence the rate of proliferation, cell differentiation and maturation.[18,25,39]

Given the similarities in the differentiation pattern between HDPC and osteoblasts-like cells, we were not

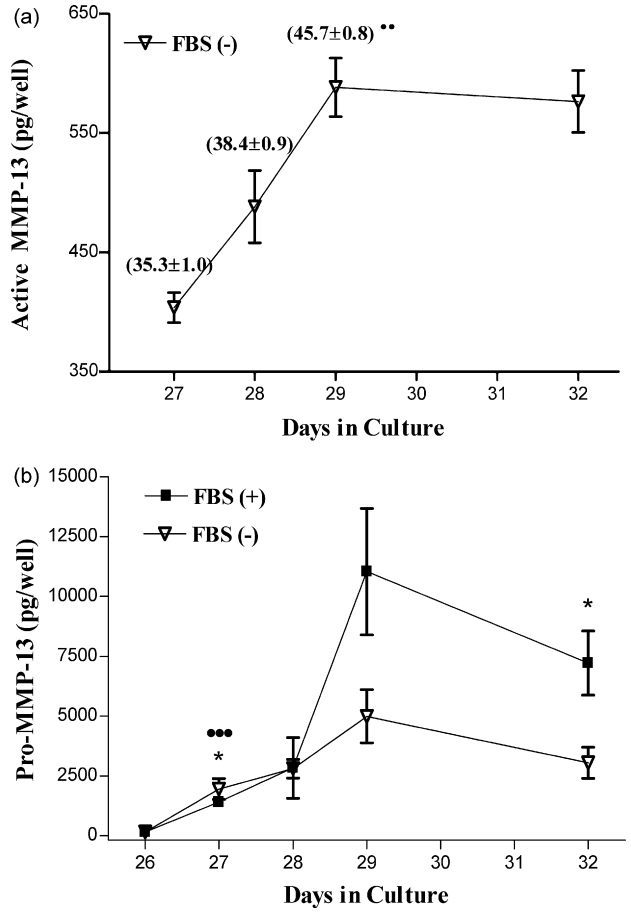

Fig. 8 – (a) Expression of active MMP-13 in FBS (−) HDPC cultures around the onset of mineralisation. MMP-13 activity was not detectable when FBS was present in the medium. Values represent the mean ± S.E.M. from six independent experimental samples. Numbers in parentheses represent active MMP-13 amounts normalised to DNA from the same samples (pg/μg DNA, mean ± S.E.M.). Bullets indicate statistical significance in normalised values within the FBS (−) group between sequential time points: $^{••}p < 0.01$. No statistical significance was found in raw active MMP-13 values (b) expression of pro-MMP-13 in FBS (+) and FBS (−) HDPC cultures at the same time points as in Fig. 7. Values represent the mean ± S.E.M. from six independent experimental samples. Asterisks denote statistical significance between the two groups and bullets indicate statistical significance within the FBS (−) group between sequential time points: $^{*}p < 0.05$; $^{•••}p < 0.001$.

surprised to find that mineralising HDPC cultures expressed significant amount of pro-MMP-13. In contrast, pro-MMP-13 production was well below the threshold of detectability in the non-mineralising group (Fig. 4). Interestingly, after mineralisation had been established, pro-MMP-13 levels dropped significantly (Figs. 4a and 7). A similar pattern of MMP-13 expression at the mRNA level was reported in mineralising rat calvarial osteoblasts, however no information was available regarding protein and/or activity levels.[40] The authors concluded that upregulation of MMP-13 expression is triggered by cessation of proliferation, since a small increase in MMP-13 mRNA was also seen in non-mineralising cultures post-confluence. Although our results are not directly comparable, pro-MMP-13 expression in HDPC cannot be associated with proliferation, since mineralising cultures demonstrated a dramatic increase in pro-MMP-13 levels between days 20 and 32, when DNA content initially increased (days 20–26) and then decreased (days 26–32) (Figs. 1 and 4).

A rather unique aspect of this study was measurement of MMP-13 activity (Fig. 7). For most cell culture studies this is difficult, since FBS contains non-specific MMP inhibitors, such as α_2-macroglobulin. Pro-MMP-13 secretion in culture supernatants correlated well with enzymatic activity, with a gradual increase for the first 3 days and a plateau for the subsequent 3-day period (Fig. 7). Correlation of the pro- and active MMP-13 forms has also been shown in osteoblasts.[13,41] Serum deprivation did result in a decrease of pro-MMP-13 expression at day 29, which proceeded to become statistically significant on day 32. This finding was consistent with the reported upregulation of collagenase levels by serum factors in osteoblasts.[42] Interestingly, serum deprivation at this stage in culture did not influence HDPC DNA content or mineralisation (Figs. 5 and 6). This allowed meaningful comparisons between the FBS (+) and FBS (−) groups. Most importantly, the daily collection of samples in this experiment allowed normalisation of MMP-13 levels (pro- and active) to DNA content. Measurement of MMP-13 in culture supernatants reflects a gradual accumulation of the enzyme since the last medium change as well as a concomitant loss due to possible internalisation and/or degradation. In contrast, DNA content represents cellular density at a specific time point. When time points are 3–6 days apart, correlation of the two is meaningless. When samples were collected daily however, normalisation is more appropriate, albeit still inaccurate. Normalised values of both pro- and active MMP-13 did increase over a critical 3-day period around the onset of mineralisation. The trend was similar for all measurements, with a 2–3-fold higher rate of increase between days 28 and 29 compared to that observed between days 27 and 28. This analysis confirmed that the peak of pro- and active MMP-13 is associated with the onset of mineralisation.

Although the association of MMP-13 with cell differentiation and matrix mineralisation has been adequately described in osteoblastic and chondroblastic cells as well as in bone and cartilage,[4,9,43–45] the mechanism for this MMP-13 action is still unclear. A plausible explanation is that terminal differentiation of cells that can produce a mineralised matrix requires proteolysis and remodelling of the ECM.[12] MMP-13 is a good candidate for this role, given the wide array of substrates it can cleave. These include collagens type I, II, IV, IX, X and XIV, as well as non-collagenous proteins, such as aggrecan, tenascin, fibronectin, fibrillin and fibromodulin.[45–48] In addition, MMP-13 may promote bioavailability of growth factors that affect cell survival and differentiation, such as TGFβ.[49] The association of MMP-13 with matrix vesicles[49] could also explain the concurrent peak of MMP-13 and ALP activity. Interestingly, MMP-13 has been associated with apoptosis of hypertrophic chondrocytes in the developing growth Plate[45] and with activation of caspace-3 in cultured chondrocytes.[50] Furthermore, factors that

promote apoptosis upregulate MMP-13 expression.[51,52] This was consistent with our findings, since the peak of pro-MMP-13 production correlated with a significant decrease in DNA content (Figs. 1 and 4). This decrease in DNA corresponds with an increase in LDH release, indicating cell death.

The findings from this study implicate for the first time a role for MMP-13 in HDPC matrix remodelling and mineralisation. MMP-13 is measurable only in mineralising cultures, where it demonstrates a striking association with the onset of mineralisation and decline in cell viability. This leads us to postulate that MMP-13 expression could reflect a critical transition stage in HDPC differentiation. Clearly, further studies are needed to explore the mechanism for this action. HDPC represent an easily accessible and reproducible *in vitro* model for these studies, with the additional advantage of a relative short transition to a mineralising state, as compared to the gradual mineralisation seen in osteoblasts. This could facilitate exploring gene expression changes associated with mineralisation as well as any role of MMP-13 in these changes.

REFERENCES

1. Woessner Jr JF. The matrix metalloproteinase family. In: Parks WC, Mecham RP, editors. *Matrix metalloproteinases*. 1st ed. San Diego: Academic Press; 1998. p. 1–15.
2. Woessner Jr JF. *Matrix metalloproteinases and TIMPS*. 1st ed. New York: Oxford Press; 2000.
3. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;**92**(8):827–39.
4. Ortega N, Behonick D, Stickens D, Werb Z. How proteases regulate bone morphogenesis. *Ann NY Acad Sci* 2003;**995**:109–16.
5. Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van Obberghen E, et al. Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem* 2003;**278**(14):11888–96.
6. Smith MF, Ricke WA, Bakke LJ, Dow MP, Smith GW. Ovarian tissue remodeling: role of matrix metalloproteinases and their inhibitors. *Mol Cell Endocrinol* 2002;**191**(1):45–56.
7. Slongo ML, Zampieri M, Onisto M. Expression of matrix metalloproteases (MMP-2, MT1-MMP) and their tissue inhibitor (TIMP-2) by rat sertoli cells in culture: implications for spermatogenesis. *Biol Chem* 2002;**383**(1):235–9.
8. Jacob SS, Shastry P, Sudhakaran PR. Monocyte-macrophage differentiation in vitro: modulation by extracellular matrix protein substratum. *Mol Cell Biochem* 2002;**233**(1–2):9–17.
9. Tuckermann JP, Pittois K, Partridge NC, Merregaert J, Angel P. Collagenase-3 (MMP-13) and integral membrane protein 2a (Itm2a) are marker genes of chondrogenic/osteoblastic cells in bone formation: sequential temporal, and spatial expression of Itm2a, alkaline phosphatase, MMP-13, and osteocalcin in the mouse. *J Bone Miner Res* 2000;**15**(7): 1257–65.
10. Winchester SK, Selvamurugan N, D'Alonzo RC. Partridge NCIPNC. Developmental regulation of collagenase-3 mRNA in normal, differentiating osteoblasts through the activator protein-1 and the runt domain binding sites. *J Biol Chem* 2000;**275**(30):23310–8.
11. Winchester SK, Bloch SR, Fiacco GJ. Partridge NCIPNC. Regulation of expression of collagenase-3 in normal, differentiating rat osteoblasts. *J Cell Physiol* 1999;**181**(3): 479–88.
12. Wu CW, Tchetina EV, Mwale F, Hasty K, Pidoux I, Reiner A, et al. Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. *J Bone Miner Res* 2002;**17**(4):639–51.
13. Mizutani A, Sugiyama I, Kuno E, Matsunaga S, Tsukagoshi N. Expression of matrix metalloproteinases during ascorbate-induced differentiation of osteoblastic MC3T3-E1 cells. *J Bone Miner Res* 2001;**16**(11):2043–9.
14. Johansson N, Saarialho-Kere U, Airola K, Herva R, Nissinen L, Westermarck J, et al. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. *Dev Dyn* 1997;**208**(3):387–97.
15. Porte D, Tuckermann J, Becker M, Baumann B, Teurich S, Higgins T, et al. Both AP-1 and Cbfa1-like factors are required for the induction of interstitial collagenase by parathyroid hormone. *Oncogene* 1999;**18**(3):667–78.
16. Jimenez MJ, Balbin M, Alvarez J, Komori T, Bianco P, Holmbeck K, et al. A regulatory cascade involving retinoic acid, Cbfa1, and matrix metalloproteinases is coupled to the development of a process of perichondrial invasion and osteogenic differentiation during bone formation. *J Cell Biol* 2001;**155**(7):1333–44.
17. Arenholt-Bindslev D, Bleeg H. Characterization of two types of human oral fibroblast with a potential application to cellular toxicity studies: tooth pulp fibroblasts and buccal mucosa fibroblasts. *Int Endod J* 1990;**23**(2):84–91.
18. Moule AJ, Li H, Bartold PM. Donor variability in the proliferation of human dental pulp fibroblasts. *Aust Dent J* 1995;**40**(2):110–4.
19. Inoue T, Chen SH, Usuda J, Morohoshi Y, Shimono M. Osteogenic activity of cells from dental pulp, periodontal ligament, bone marrow and muscle in vitro: an ultrastructural study and alkaline-phosphatase activity. *Bull Tokyo Dent Coll* 1992;**33**(1):7–12.
20. Alliot-Licht B, Hurtrel D, Gregoire M. Characterization of alpha-smooth muscle actin positive cells in mineralized human dental pulp cultures. *Arch Oral Biol* 2001;**46**(3):221–8.
21. Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H. Odontoblast differentiation of human dental pulp cells in explant cultures. *Calcified Tissue Int* 2000;**66**(2):129–38.
22. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003;**18**(4):696–704.
23. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;**81**(8):531–5.
24. Tsukamoto Y, Fukutani S, Shin-Ike T, Kubota T, Sato S, Suzuki Y, et al. Mineralized nodule formation by cultures of human dental pulp-derived fibroblasts. *Arch Oral Biol* 1992;**37**(12):1045–55.
25. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000;**97**(25):13625–30.
26. Shi S, Robey PG, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone* 2001;**29**(6):532–9.
27. Takeda K, Oida S, Goseki M, Iimura T, Maruoka Y, Amagasa T, et al. Expression of bone morphogenetic protein genes in the human dental pulp cells. *Bone* 1994;**15**(5):467–70.
28. Palosaari H, Pennington CJ, Larmas M, Edwards DR, Tjaderhane L, Salo T. Expression profile of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in mature human odontoblasts and pulp tissue. *Eur J Oral Sci* 2003;**111**(2):117–27.
29. Sulkala M, Paakkonen V, Larmas M, Salo T, Tjaderhane L. Matrix metalloproteinase-13 (MMP-13, collagenase-3) is

highly expressed in human tooth pulp. *Connect Tissue Res* 2004;**45**(4–5):231–7.
30. Gagari E, Rand MK, Tayari L, Vastardis H, Sharma P, Hauschka PV, et al. Expression of stem cell factor and its receptor, c-kit, in human oral mesenchymal cells. *Eur J Oral Sci* 2006;**114**(5):409–15.
31. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, et al. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds [see comment]. *Nature* 1993;**364**(6438):626–32.
32. Andersch MA, Szczypinski AJ. Use of p-nitrophenyl phosphate substrate in determination of serum acid phosphatase. *Am J Clin Pathol* 1947;**17**(571).
33. Lowry OH, Roberts NR, Wu ML, Hixon WS, Crawford EJ. The quantitative histochemistry of brain. II. Enzyme measurements. *J Biol Chem* 1954;**207**(1):19–37.
34. Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). *J Biol Chem* 1995;**270**(16): 9420–8.
35. Aubin JE, Triffitt JT. Mesenchymal stem cells and osteoblast differentiation. In: Bilezikian JP, Lawrence GR, Gideon AR, editors. *Principles of bone biology*. 2nd ed. San Diego: Academic Press; 2002.
36. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* 1990;**143**(3):420–30.
37. Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. *Endocr Rev* 1993;**14**(4):424–42.
38. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 1988;**136**: 42–60.
39. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003;**100**(10):5807–12.
40. Winchester SK, Bloch SR, Fiacco GJ, Partridge NC. Regulation of expression of collagenase-3 in normal, differentiating rat osteoblasts. *J Cell Physiol* 1999;**181**(3):479–88.
41. Fratzl-Zelman N, Glantschnig H, Rumpler M, Nader A, Ellinger A, Varga F. The expression of matrix metalloproteinase-13 and osteocalcin in mouse osteoblasts is related to osteoblastic differentiation and is modulated by 1,25-dihydroxyvitamin D3 and thyroid hormones. *Cell Biol Int* 2003;**27**(6):459–68.
42. Puccinelli JM, Omura TH, Strege DW, Jeffrey JJ. Partridge NCIPNC. A serum factor promotes collagenase synthesis by an osteoblastic cell line. *J Cell Physiol* 1991;**147**(3):505–13.
43. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, et al. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 1999;**99**(1):81–92.
44. Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, et al. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Natl Acad Sci USA* 2000;**97**(8):4052–7.
45. Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, et al. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* 2004;**131**(23):5883–95.
46. Ashworth JL, Murphy G, Rock MJ, Sherratt MJ, Shapiro SD, Shuttleworth CA, et al. Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodelling. *Biochem J* 1999;**340**(Pt 1):171–81.
47. Heathfield TF, Onnerfjord P, Dahlberg L, Heinegard D. Cleavage of fibromodulin in cartilage explants involves removal of the N-terminal tyrosine sulfate-rich region by proteolysis at a site that is sensitive to matrix metalloproteinase-13. *J Biol Chem* 2004;**279**(8):6286–95.
48. Knauper V, Cowell S, Smith B, Lopez-Otin C, O'Shea M, Morris H, et al. The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction. *J Biol Chem* 1997;**272**(12):7608–16.
49. D'Angelo M, Billings PC, Pacifici M, Leboy PS, Kirsch T. Authentic matrix vesicles contain active metalloproteases (MMP). A role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. *J Biol Chem* 2001;**276**(14):11347–53.
50. Haisch A, Marzahn U, Mobasheri A, Schulze-Tanzil G, Shakibaei M. Development and phenotypic characterization of a high density in vitro model of auricular chondrocytes with applications in reconstructive plastic surgery. *Histol Histopathol* 2006;**21**(5):467–76.
51. Ijiri K, Zerbini LF, Peng H, Correa RG, Lu B, Walsh N, et al. A novel role for GADD45beta as a mediator of MMP-13 gene expression during chondrocyte terminal differentiation. *J Biol Chem* 2005;**280**(46):38544–55.
52. Sabatini M, Rolland G, Leonce S, Thomas M, Lesur C, Perez V, et al. Effects of ceramide on apoptosis, proteoglycan degradation, and matrix metalloproteinase expression in rabbit articular cartilage. *Biochem Biophys Res Commun* 2000;**267**(1):438–44.