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Comparative gene expression profile analysis between native human odontoblasts and pulp tissue

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Abstract

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Aim To undertake a large-scale analysis of the expression profiles of native human pulp tissue and odontoblasts, and search for genes expressed only in odontoblasts.

Methodology Microarray was performed to pooled pulp and odontoblasts of native human third molars and to pooled +/− TGF-β1 cultured pulps and odontoblasts (137 teeth). The repeatability of microarray analysis was estimated by comparing the experimental pulp samples with expression profiles of two pulp samples downloaded from the GEO database. The genes expressed only in the experimental pulp samples or in odontoblasts were divided into categories, and the expression of selected odontoblast-specific genes of extracellular matrix (ECM) organization and biogenesis category was confirmed with RT-PCR and Western blot.

Results A 85.3% repeatability was observed between pulp microarrays, demonstrating the high reliability of the technique. Overall 1595 probe sets were positive only in pulp and 904 only in odontoblasts. Sixteen expressed sequence tags (ESTs), which represent transcribed sequences encoding possibly unknown genes, were detected only in odontoblasts; two consistently expressed in all odontoblast samples. Matrilin 4 (MATN4) was the only ECM biogenesis and organization related gene detected in odontoblasts but not in pulp by microarray and RT-PCR. MATN4 protein expression only in odontoblasts was confirmed by Western blot.

Conclusions Pulp tissue and odontoblast gene expression profiling provides basic data for further, more detailed protein analysis. In addition, MATN4 and the two ESTs could serve as an odontoblast differentiation marker, e.g. in odontoblast stem cell research.

Keywords: collagen, gene expression analysis, matrilins, odontoblast, pulp tissue.

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Introduction

Odontoblasts are hard tissue-forming cells, responsible for the formation of dentine, whilst the adjacent pulp tissue is fibroblast-derived soft connective tissue. Knowledge of differential gene expression of pulp and odontoblasts might give insight to the regulatory mechanisms of these spatially related but functionally diverse cells. Comprehensive screens of expression differences in these tissues have been, however, extremely rare. Suppression subtractive hybridization has been used to compare the gene expression at large scale between cultured fibroblastic pulp cells and cultured odontoblast-like cells (Buchaille *et al.* 2000). The same method was used to find genes expressed differentially or predominantly in rat odontoblasts, by subtracting the common transcripts in odontoblasts, osteoblasts and pulp cells (Dey *et al.* 2001). Shi *et al.* (2001) compared gene expression profiles of bone

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marrow stem cells and dental pulp stem cells using microarray. cDNA microarray and 2D electrophoresis has been used to compare healthy and carious human dental pulp tissues (Pääkkönen *et al.* 2005). However, only a few slight changes at mRNA level could be detected, probably because only teeth with small or moderate carious lesions were used, and the samples thus contained relatively large amounts of unaffected tissue (Pääkkönen *et al.* 2005). McLachlan *et al.* (2005) used microarray to compare healthy and carious pulp tissues using teeth with varying degrees of lesion size, from enamel involvement only to deep dentinal lesions. However, as their samples contained teeth with extensive pulp inflammation, the detected expression most likely represents the gene expression of inflammatory cells.

The aim of this study was to compare the gene expression profiles between healthy native odontoblasts and pulp tissue, to identify genes expressed solely in either sample. Additionally, the expression profile of pulp tissue acquired in the study was compared with the profiles of two healthy pulp tissue samples downloaded from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/projects/geo/) (McLachlan *et al.* 2005) to examine similarities and differences between separate hybridizations by different laboratories. TGF-β1 has been shown to be associated with regulation of odontoblast differentiation and tertiary dentine formation in response to the dental injury (Tziafas *et al.* 2000). Thus, the second aim was to analyse the effects of TGF-β1 on expression levels of expressed sequence tags (ESTs), which represent transcribed sequences encoding possibly unknown genes, detected only in odontoblasts using human pulp tissue and odontoblast cultures.

Material and methods

Tooth sample collection

Intact third molars were removed from patients attending the Oulu Health Care Centre Dental Specialty Care Unit. All the patients were healthy young (18–25 years) adults. The study was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District. The molars were used for the experiments after the patient's informed consent, following the guidelines of the Faculty of Medicine at the University of Oulu for the use of human samples in research.

The teeth were prepared for sample collection or culturing immediately after the extraction as described previously in detail (Palosaari *et al.* 2000). Briefly, the teeth were swabbed with 70% alcohol, and soft tissue and cementum removed. A 2-mm deep horizontal groove was cut around the root about 5 mm apically from the cemento-enamel junction with a diamond disk. The root was dissected from the crown with pliers, and the crowns were placed into sterile culture medium. The rest of the procedures were performed in a laminar flow tissue culture hood under sterile conditions.

To collect the native odontoblast and pulp tissue mRNA samples, the pulps were removed with forceps, using a gentle pulling force perpendicular to tooth axis. This pulp removal technique has been shown to separate pulp tissue from the odontoblasts, leaving the intact odontoblast layer to cover the pulp chamber (Tjäderhane *et al.* 1998). The pulp tissue was placed into Eppendorf tubes containing 60 μL of Trizol® Reagent solution (Gibco BRL, Roskilde, Denmark). The odontoblasts lining the pulp chamber walls were covered with a small amount of Trizol® Reagent to prevent RNA degradation, and the cells were immediately scraped off with a sterile excavator and stored into a final volume of 30 μL of Trizol® Reagent.

Pulp and odontoblast tissue cultures

To analyse the effect of TGF-β1, which is the most extensively studied cytokine in pulp–dentine complex, on gene expression, pulp tissues were cultured with and without 1 ng mL^{-1} TGF-β1 (Sigma, Saint Louis, MO, USA) for 1, 5, 24 and 48 h (n = 5–7 in each group), and odontoblasts for 1 h (n = 37, n = 38) and 24 h (n = 19, n = 19) in serum free OPTI-MEM 1 (Invitrogen, Paisley, UK) culture medium supplemented with 100 IU mL^{-1} penicillin–streptomycin (Biowhittaker Europe, Verviers, Belgium), 0.25 μg mL^{-1} Fungizone (Gibco-BRL, Paisley, UK) and 50 μg mL^{-1} vitamin C (Sigma). The tissues for culturing were obtained and the samples were treated for RNA analysis as described above for native samples. The detailed procedures of pulp and odontoblast tissue cultures have been described previously (Tjäderhane *et al.* 1998, Palosaari *et al.* 2000).

Mouse bone samples

Twenty tibia of 4-day-old mice (provided by Outi Lahti, Department of Anatomy and Cell Biology, University of Oulu) were frozen in liquid nitrogen. The frozen tissues

were pooled and pulverized, and proteins were eluted with buffer containing 50 mmol L^{-1}TRIS, 10 mmol L^{-1} $CaCl_2$, 150 mmol L^{-1} NaCl and 0.05% Brij 35 by incubation for 1 h at 4 °C.

Isolation of mRNA from pulp and odontoblast samples

The pulp tissue and odontoblast samples were pooled and total RNA for microarray and RT-PCR was isolated from the pools of native pulp tissues and odontoblasts, as well as pooled cultured pulp tissues and odontoblasts, using Trizol® reagent and protocol (GIBCO-BRL, Gaithersburg, MD, USA), and further purified by Qiagen RNeasy Mini Kit (Hilden, Germany) according to the manufacturer's instructions.

Microarray and data analysis

Experimental procedures for GeneChip (Affymetrix, Santa Clara, CA, USA) were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual. Affymetrix HGU133A array, containing over 22 000 probe sets that represent over 14 500 well-characterized genes, was used for analysis of pooled native and cultured pulp tissue samples, and Affymetrix HGU133plus 2.0 array (over 54 000 probe sets and 38 500 genes) for pooled native and cultured odontoblast samples. The samples were hybridized once.

Probe sets detecting expression (detection *P*-values under 0.05) only in either native pulp tissue or native odontoblasts according to raw data analysis with Affymetrix GeneChip Operating Software, which uses one-sided Wilcoxon's signed rank test to calculate the *P*-value and discrimination value (τ) of 0.015 to filter away the probe sets detecting nonspecific expression, were selected for further analysis. These probe sets were further analysed with Onto-Express (http://vortex.cs.wayne.edu) software to divide the probe sets into categories according to the Gene Ontology classifications (http://www.geneontology.org). Some interesting categories [cell differentiation, extracellular matrix (ECM) organization and biogenesis, apoptosis, cell adhesion and cell–cell signalling] were further analysed by manually checking all the probes representing genes of these categories to exclude those represented by several probes, with conflicting expression profiles.

Expressed sequence tags are short sequences of transcribed nucleotide sequences that may represent previously unknown genes coding for new proteins and are valuable tool for discovery of new genes. Probe sets detecting ESTs positive in native odontoblasts but not in native pulp tissue were further analysed by checking their expression in pulp tissues cultured with and without TGF-β1. Next the ESTs represented by the remaining probe sets were checked from NCBI Entrez Nucleotide (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide&cmd=search&term=) and Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene) databases to exclude the ones representing ESTs previously confirmed to represent known genes or pseudogenes. Then the behaviour of the remaining probe sets in +/− TGF-β1 cultured odontoblasts was checked to estimate the consistency of their expression in odontoblasts. As above, ESTs, which were represented by probe sets that were positive according to raw data analysis with Affymetrix GeneChip Operating Software and had detection *P*-value under 0.05, were considered to be expressed in sample.

As dentine sialophosphoprotein (DSPP) is traditionally considered to be odontoblast specific, microarray data of DSPP gene expression in native odontoblasts and pulp tissue as well as in odontoblasts cultured for 1 and 24 h and pulp tissues cultured for 1, 5, 24 and 48 h was manually checked similarly as ESTs. Due to different probe densities on the microarrays (HGU133A and HGU133 Plus 2.0), direct comparison of expression levels of individual genes between pulp tissue and odontoblasts could not be performed. For indirect comparison of DSPP expression levels between pulp tissue and odontoblasts, the intensities of microarrays used to study native pulp and native odontoblasts were scaled to the same level using housekeeping controls (n = 16). The spike controls, which detect exogenous transcripts added to sample and are used to monitor hybridization, washing and staining of microarray, were excluded from the scaling. Then the DSPP mRNA expression ratio was calculated from the scaled intensity values of probe sets detecting DSPP expression on the microarrays, allowing the comparison between native pulp and native odontoblasts.

Microarray data download from gene expression omnibus and processing

Data of expression profile of two healthy human dental pulp samples under number GSE1629 (McLachlan *et al.* 2005), obtained using same

Affymetrix HGU133A array as the present data, was downloaded from GEO (http://www.ncbi.nlm.nih.gov/projects/geo/). Numbers of positive probe sets were compared between the GSE1629 samples and the experimental pulp tissue samples so that the amounts of probe sets found only in one, in two and in all three samples were calculated. Probe sets detecting expression only in the experimental samples were further checked to exclude genes with conflicting probe sets, and the resulting list of genes was further divided into GO categories. Percentual agreement levels between individual samples were calculated from amounts of probe sets agreeing and disagreeing in presence or absence of expression between two samples.

RT-PCR of collagen XI α1 and matrilin 4

The same RNA pools of native odontoblasts and pulp tissue as above were used for RT-PCR using AMV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCR reaction was carried out in a mixture containing 200 μmol L^{-1} dNTP (Promega), 300 nmol L^{-1} primers, 2 mmol L^{-1} $MgCl_2$ and 2.6 U polymerase (Roche Molecular Biochemicals, Mannheim, Germany) using initial denaturation of 2 min at 94 °C; then for 10 cycles denaturation of 15 s at 94 °C, annealing of 30 s at 56 °C and extension of 45 s at 72 °C, for the cycles after 10 extension time was extended by 5 s per cycle. Number of cycles used for PCR was 32 for both collagen XI α1 (COL11A1) and matrilin 4 (MATN4). GenBank accession codes (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and primers used are shown in Table 1. Primers used for COL11A1 were designed to distinguish the splicing variants. Intensities of resulting product bands were calculated using ScionImage software (Scion Corporation, Frederick, MA, USA) and the intensities of COL11A1 and MATN4 were compared with β-actin to determine the relative amounts. The PCR analyses were done in duplicates.

Table 1 The GenBank accession codes and primers used for genes analysed with semi-quantitative RT-PCR

Gene	Accession codes	Primers
COL11A1	AF101079	forward: cca gac tgt gac tct tca gc reverse: tgc tgg ttc tcc ttt ctg tc
MATN4	NM_003833	forward: cgt aga gtc ctt cga cct ca reverse: cac aca ctg gaa ctc aca gc
β-actin	X00351	forward: cca agg cca acc gcg aga aga tga c reverse: agg gta cat ggt ggt gcc gcc aga c

Western blot of matrilin 4

Total protein extracts for Western blot were isolated from the pools of native pulp tissue and odontoblasts used for microarray analysis according to the Trizol® reagent protocol. Mouse bone (tibia of 4 days old mouse), known to express high amounts of MATN4 (Klatt *et al.* 2001) was used as a positive control for Western blot. Ten microgram aliquots of proteins extracted from pools of human native pulp tissue and odontoblasts and mouse bone samples, as described above, were treated with 2-mercaptoethanol for 3 min at 100 °C to achieve reducing conditions, run on 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), and the gel was stained with Coomassie brilliant blue to allow detection of albumin and scanned. Then the staining was removed by incubating the gel overnight with solution containing 30% of ethanol and 10% of acetic acid. Next, the proteins were transferred to Immobilon P membrane (Millipore Corp., Bedford, MA, USA) and the membrane was handled using reagents and according to manufacturer's instructions of ECL Western blot detection reagents and analysis system (Amersham Biosciences, Buckinghamshire, UK). The primary antibody used was rabbit polyclonal antibody against mouse MATN4 that cross-reacts with the human protein (kind gift from Dr Raimund Wagener, Cologne, Germany) at 1 : 500 dilution overnight at 4 °C. Analysis was performed in triplicates.

The membranes were stripped according to the instructions of ECL Western blot detection reagents (Amersham Biosciences) and reprobed with monoclonal antibody against β-actin (Novus Biologicals, Littleton, CO, USA) at 1 : 2000 dilution according to the ECL protocol (Amersham Biosciences).

Intensities of stained albumin as well as immunoreactive MATN4 and β-actin bands were determined with ScionImage software (Scion Corporation), and relative amounts of MATN4 against albumin and β-actin were calculated. MATN4/albumin and MATN4/β-actin ratios were normalized by giving ratio in mouse bone sample value 1. Mean and standard deviation values of three separate analyses were calculated.

Results

Comparison of pulp sample with data extracted from GEO database

Comparison of the experimental pulp tissue microarray data with pulp microarray data presented in GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) under number GSE1629, revealed striking similarities between the three samples (Fig. 1a). 10 255 (46.2% of total number of probe sets) were positive in all three samples, whereas 8658 probe sets (40.0%) were detected in none of them. 2137 probe sets (9.6%) were positive in two of the samples, and 2031 (9.1%) in one of the samples. Percentual level of agreement between the experimental pulp sample and either of samples of GSE1629 was 85.3%, whilst between individual GSE1629 samples it was 88.2% (Table 2). Only 430 probe sets (<2% of total of over 22 000) were detected solely in the experimental sample.

Comparison of pulp and odontoblast samples with microarray

The comparison of pulp tissue and odontoblast gene expression profiles revealed 9719 positive probe sets (43.7% of total number of probe sets in microarray used for pulp sample) both in pulp tissue and odontoblasts. In addition, the analyses revealed 1595 positive probe sets (7.2% of total number of probe sets) in pulp but not in odontoblasts, and 904 positive probe sets (4.1%) only in odontoblasts. The complete lists of the probe sets positive either in pulp tissue or in odontoblasts are presented as supplementary data (Table S1 for the pulp tissue and Table S2 for odontoblasts). The complete microarray data of pulp tissue and odontoblasts is available at the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) under number GSE8694.

Next, the transcripts present only in pulp or only in odontoblasts were divided into categories according to the GO classifications (http://www.geneontology.org)

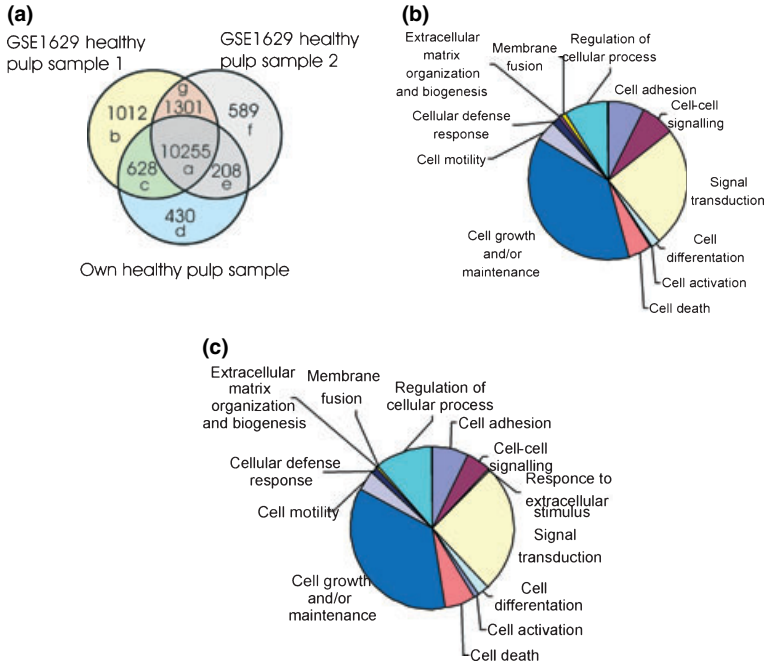

Figure 1 (a) Venn diagram demonstrating the numbers of probe sets detecting gene expression in our own pulp sample and in healthy samples 1 and 2 loaded from GEO (http://www.ncbi.nlm.nih.gov/projects/geo/) under number GSE1629. 430 probe sets were detected only in our sample, and 1012 and 589 only in GSE1629 healthy sample 1 and 2 respectively. 10 255 probe sets were detected in all samples and 8658 were detected in none of them. Letters a to g refer to symbols used in Table 2 to calculate percentual agreement levels between the samples. (b) Gene Ontology (GO) classification of transcripts present only in odontoblasts according to cellular process. (c) GO classification of transcripts present only in pulp tissue according to cellular process. The GO classifications obtained using Onto-Express software (http://vortex.cs.wayne.edu).

Table 2 Percentual agreement level between the pulp samples (GSE1629 samples 1 and 2, and our own pulp sample). Letters from a to g refer to Fig. 1 in which distribution of numbers of probe sets between the samples is presented. Absent refers to amount of probe sets that detected no expression in any of the samples

Sample	Agreeing probe sets	Disagreeing probe sets	% agreement
GSE1629 1 and own	a, c, f, absent	b, d, e, g	85.3
GSE1629 2 and own	a, b, e, absent	c, d, f, g	85.3
GSE1629 1 and 2	a, d, g, absent	b, c, f, e	88.2

(Fig. 1b,c). Distribution of genes into different GO categories was very similar in pulp tissue and odontoblasts.

Some interesting categories, including cell differentiation, ECM organization and biogenesis, apoptosis, cell adhesion and cell–cell signalling were selected for further analysis, in which all the probes representing genes of those categories were checked to exclude ones with conflicting probes. Altogether in these categories, 44 genes were solely expressed in odontoblasts and 92 only in pulp tissue. The genes that were thus confirmed to be expressed solely in odontoblasts or in pulp tissue are presented as supplementary data (Table S3).

Expressed sequence tags

Microarray comparison of native pulp tissue and odontoblasts revealed several ESTs and genes with unknown function detectable in native odontoblasts but not in native pulp tissue. After the exclusion of ESTs representing previously known genes and pseudogenes, 16 odontoblast-expressed ESTs that were undetectable in any of the cultured pulp samples remained (Table 3). Two of the ESTs were consistently expressed in all of the cultured odontoblast samples (Table 3).

Expression of DSPP in odontoblasts and pulp tissue according to microarray

Dentine sialophosphoprotein, which is commonly accepted as odontoblast marker, was detected in native odontoblasts and in all of the cultured odontoblasts, but no expression change in response to TGF-β1 treatment was detected. DSPP was also detected in native pulp tissue and in all of the cultured pulp samples, and TGF-β1 had no marked effect on its expression. Indirect comparison of microarray data of native odontoblasts and pulp tissue revealed 34.7 times higher DSPP mRNA level in odontoblasts than in pulp tissue.

Table 3 EST sequences positive in native odontoblasts (OB) but not in native pulp tissue or any of the cultured pulp samples, and the expression changes during odontoblast culture and in response to TGF-β1 treatment as detected with microarray

Accession	OB native	OB 1 h C	OB 1 h T	OB 24 h C	OB 24 h T	
AA071454	X	X	X			++
AA521034	X	X	X	X		++
AA601208	X	X	X	X	X	+++
AB020695	X	X	X		X	++
AF052117	X					+
AF070571	X	X	X			++
AI740515	X	X	X	X	X	+++
AK021987	X	X	X			++
AK025194	X					+
AL049278	X	X	X			++
AL049280	X	X				++
AL050043	X			X		++
AL109705	X				X	++
AW969803	X	X	X			++
L34409	X					+
U79300	X	X	X			++

Accession refers to GenBank accession codes. X indicates detected expression, and blank indicates that no expression was detected. Number of + symbols show estimation of importance of EST to function of odontoblast based on consistency of expression during the odontoblast cultures and in response to TGF-β1 cultures. +, EST was detected only in native odontoblasts; ++, EST was detected in native and at least one of the cultured samples; +++, EST was detected in native odontoblasts and in all of the cultured odontoblast samples.

Confirmation of COL11A1 and MATN4 expression with RT-PCR

With the present microarray only two ECM organization and biogenesis-related genes, COL11A1 and MATN4, were detected in odontoblasts but not in pulp tissue and were selected for further study. RT-PCR confirmed the expression of MATN4 only in odontoblasts (Fig. 2). RT-PCR also showed the expression of 354 bp COL11A1 splicing variant in odontoblasts. However, in pulp tissue the expression of 354 and 608 bp variants of COL11A1 were observed. Relative comparison to β-actin revealed that the amount of 354 bp variant expressed in both pulp tissue and odontoblasts was in pulp tissue only approximately one-third of that observed in odontoblasts.

COL11A1 and MATN4 expression according to data extracted from the GEO database

According to the data of two healthy pulp samples extracted from the GEO database under number

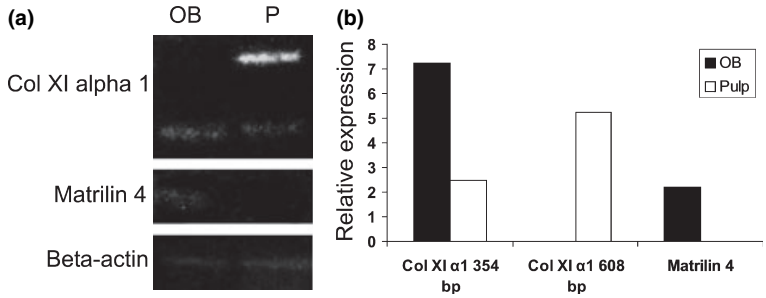

Figure 2 (a) RT-PCR analysis showing expression of one splicing variant of collagen XI α1 in odontoblasts, two variants in pulp tissue, and expression of matrilin 4 only in odontoblasts. (b) Relative expression calculated against β-actin expression. Intensities of bands were calculated using ScionImage software and intensities of collagen XI α1 and matrilin 4 and were compared with β-actin to determine the relative amounts. The analyses were done in duplicates and produced similar results.

GSE1629, COL11A1 was expressed in pulp tissue in both samples. MATN4 expression was not detected in either of the two GSE1629 samples.

Western blot of MATN4

As MATN4 mRNA was the sole ECM organization and biogenesis-related mRNA confirmed in odontoblasts but not in pulp tissue, the presence of MATN4 protein in both tissues was analysed with Western blot. MATN4 was clearly detected in native odontoblast but not in native pulp tissue sample, thus confirming the results obtained by mRNA analyses (Fig. 3a). Amounts of MATN4 in human odontoblasts were calculated relative to albumin and β-actin (Fig. 3b,c). According to both comparisons (MATN/albumin and MATN4/β-actin), MATN4 protein amount was lower in odontoblasts than in bone of 4-day-old mouse.

Discussion

Comparison of the experimental healthy pulp microarray data with data of two healthy pulp samples downloaded from GEO (http://www.ncbi.nlm.nih.gov/projects/geo/) under number GSE1629 revealed striking similarities between the samples. The experimental pulp tissue sample did not differ from the ones downloaded from GEO (similarity of 85.3%) more than they differed from each other (similarity 88.5%). Thus, the reproducibility of the method even in different laboratories is high and multiple hybridizations are unnecessary. Additionally, as the aim of this study was the qualitative search for genes present solely either in native odontoblasts or in native pulp tissue, the threshold of significant change was high, thus reducing the need for repeated hybridizations. The qualitative nature of the analysis also enabled the use of different microarrays for pulp tissue and odontoblasts.

Distribution of the genes expressed only in the experimental pulp tissue or odontoblast sample into categories was surprisingly similar despite the functional diversity of the tissues and the fact that pulp consists of several cell types. Microarray analysis also revealed 16 probe sets representing ESTs that were positive in native odontoblasts but not in native pulp tissue or in any of the cultured pulp samples, two of them consistently expressed in all cultured odontoblast samples. The genes represented by these ESTs and proteins they code for are previously unknown. Since to date their expression has not been detected in any tissues, they may be odontoblast specific and may have a distinct role in the normal function of odontoblasts.

Expression of DSPP that has been traditionally considered to be odontoblast specific was detected in all of the odontoblasts samples, but also in all of the pulp samples, with no marked effect of TGF-β1. The detection of DSPP in pulp tissue is consistent with earlier finding of dentine sialoprotein (DSP) encoded by DSPP gene in rat pulp tissue (Baba *et al.* 2004). DSPP and DSP protein have also been detected, e.g. in alveolar bone, cellular cementum and periodontium, but at lower levels than in odontoblasts (Baba *et al.* 2004). Additionally, they have been detected at low levels in nondental tissues, rodent long bone and mouse inner ear (Xiao *et al.* 2001, Qin *et al.* 2002). Thus, the present findings support the earlier results showing that DSPP is not exclusively odontoblast specific.

As odontoblasts are hard tissue-forming cells responsible for dentine formation, major differences, especially in the expression of genes related to ECM organization and biogenesis, were expected. However, surprisingly

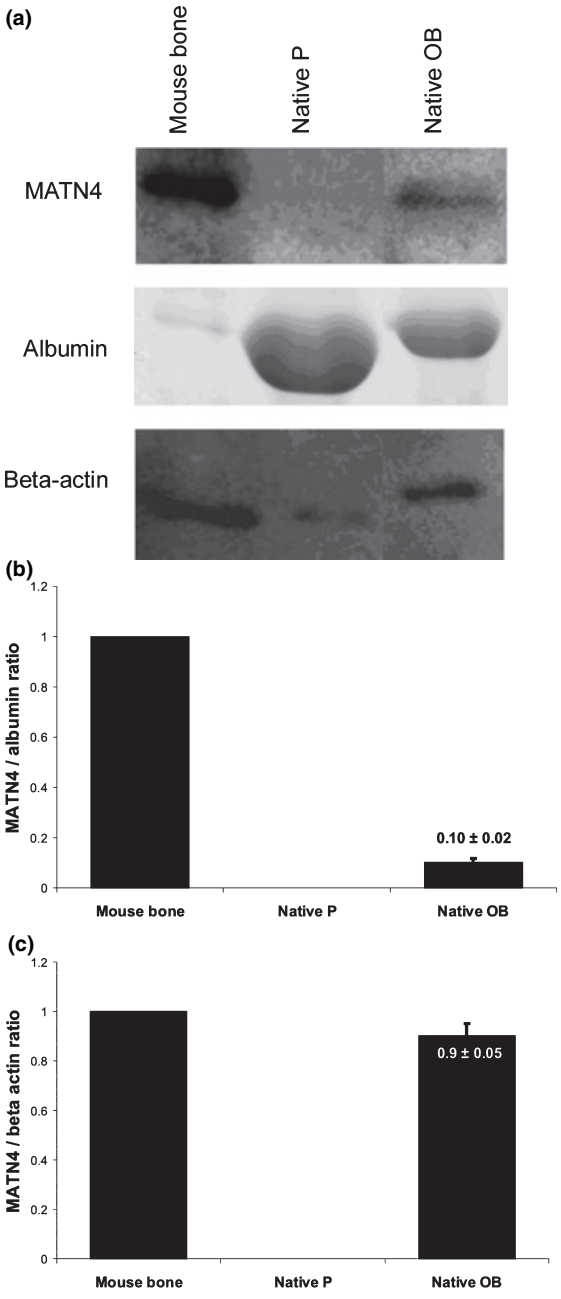

Figure 3 Production of matrilin 4 protein by human native pulp tissue and odontoblasts detected with Western blot. (a) Western blot done in reducing conditions revealed 68.5 kDa of matrilin 4 (MATN4) in native odontoblasts (OB) but not in native pulp tissue (P). Mouse bone (tibia) extract was used as a positive control. Both 66 kDa albumin (method detailed at Materials and methods) and immunoreactive 42 kDa β-actin bands (detailed at Materials and methods) were used to demonstrate the protein amount loaded of each sample to the gel. (b) Amount of immunoreactive MATN4 relative to amount of albumin. (c) Amount of immunoreactive MATN4 relative to β-actin. Intensities of stained albumin, immunoreactive MATN4 and immunoreactive β-actin were determined with ScionImage (ScionCorp). MATN4/albumin and MATN4/β-actin ratios in mouse bone were given value 1, and ratios in odontoblast samples were compared with these values. The bars represent mean and standard deviation (SD) values in detected MATN4 amounts of three analyses, and numerical values of mean and SD values are shown over the bars (b) or inside them (c).

few differences were detected, including the expression of COL11A1 and MATN4 detected only in odontoblasts. RT-PCR confirmed expression of MATN4 only in odontoblasts. It also showed expression of one splicing variant of COL11A1 in odontoblasts and two variants in pulp. The variant found in odontoblasts was also found in pulp, but in lower amount. The other variant found in pulp with RT-PCR may be undetectable with microarray used for this study. Data of two healthy pulp samples downloaded from GEO database under number GSE1629, revealed COL11A1 expression in both samples, contradicting the present result. This may be explained by differences between teeth (caused by, for example, genetic and environmental variations) used for studies and by slight differences in microarray methodology. No MATN4 expression was detected in either of samples of GSE1629, supporting the present finding that MATN4 is odontoblast specific in dentine–pulp complex.

Using Western blot MATN4 protein was clearly detected in native odontoblasts but not in native pulp, thus confirming the findings of microarray and RT-PCR. Difference between MATN4/albumin and MATN4/β-actin ratios were detected, but different amounts of β-actin mRNA have been detected in different types of mouse tissues (Erba *et al.* 1988). The same variation is also probable between mouse bone and odontoblasts. Albumin, on the other hand, is a protein that comprises half of the blood protein content. As both bone and pulp tissues contain blood vessels and odontoblast layer contains capillaries, the amount of albumin in the samples is prone to variation. Thus the MATN4/albumin and MATN4/β-actin ratios show only estimates of relative amounts of MATN4 in mouse bone and human native odontoblasts.

There are four known matrilins belonging to the superfamily of von Willebrand factor A containing proteins. The specific function of matrilins is unclear but they have been shown to interact with both collagenous and noncollagenous matrix proteins

(Piecha *et al.* 2002, Wiberg *et al.* 2003) suggesting function as general adaptor proteins in the ECM. MATN4 exists in three splicing variants (Wagener *et al.* 1998), and its expression has been detected in several tissues including bone and cartilage (Klatt *et al.* 2001). All matrilins are expressed during mouse limb development (Segat *et al.* 2000, Klatt *et al.* 2001) suggesting that they have an important role in endochondral bone formation. MATN4 protein has been indicated in primordium of incisors in mouse and in incisors of newborn mouse (Klatt *et al.* 2001, 2002). Expression of MATN4 mRNA and existence of MATN4 protein in odontoblasts but not in pulp may indicate a specific function in dentine formation. To our knowledge, this is the first time that MATN4 gene and protein expression has been detected in mature odontoblasts.

Collagen type XI (COL11) is a minor cartilage constituent, belonging to the family of fibrillar collagens (Yoshioka & Ramirez 1990), and is also expressed at low levels in noncartilaginous tissues during development (Lui *et al.* 1995, Yoshioka *et al.* 1995). It is heterotrimer consisting of three polypeptides, α1, -2 and -3 (Morris & Bachinger 1987). α1 polypeptide exists in six splicing variants (Oxford *et al.* 1995). Expression of the splicing variants varies between cell and tissue types, and is developmentally regulated in foetal rat cartilage, where it plays a role in endochondral ossification by regulating the formation of collagen fibrillar network (Davies *et al.* 1998, Morris *et al.* 2000). The presence of COL11 protein in reparative dentine has been indicated (Magloire *et al.* 1992). As COL11A1 has a role in bone formation, its expression in odontoblasts most likely indicates its function in dentine formation. Validation of this function would require further analysis at protein level, but antibody specific for α1 chain was not available.

Conclusions

This study provides novel information regarding gene expression differences between native human pulp tissue and odontoblasts. Complete lists of differentially expressed genes can be found as supplements, and the complete microarray data of both samples is available in the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) under series number GSE8694. Comparison of the present pulp sample with the data downloaded from the GEO database under number GSE1629 revealed very similar expression patterns, thus providing more reliable database of pulp expression. As both MATN4 and COL11A1 have been shown to participate in bone formation, their expression in odontoblasts could indicate function in dentine formation. MATN4, present only in odontoblasts but not in cultured pulp tissue, could serve as marker gene for odontoblast differentiation from pulp stem cells. Identification of the presently unknown genes and proteins they code for represented by ESTs and expressed only in odontoblasts may in the future reveal previously unknown and potentially odontoblast-specific proteins with important roles in odontoblast function.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Probe sets detecting expression in pulp tissue but not in odontoblasts. Probe set ID refers to Affymetrix identification code for each probe set; Title an Symbol refer to the Human Gene Nomenclature Database (http://www.gene.ucl.ac.uk/cgi bin/nomenclature/searchgenes.pl); Biological Process, Molecular Function and Cellular Component refer to Gene Ontology (GO) terms (http://www.geneontology.org) and pathway refers to GenMapp database of functional pathways (http://www.genmapp.org).

Table S2. Probe sets detecting expression in odontoblasts, but not in pulp tissue. Probe set ID refers to Affymetrix identification code for each probe set; Title an Symbol refer to the Human Gene Nomenclature Database (http://www.gene.ucl.ac.uk/cgi bin/nomenclature/searchgenes.pl); Biological Process, Molecular Function and Cellular Component refer to Gene Ontology (GO) terms (http://www.geneontology.org) and pathway refers to GenMapp database of functional pathways (http://www.genmapp.org).

Table S3. Genes expressed differentially in odontoblasts and pulp tissue in selected Gene Ontology categories. The probe sets detecting expression only either in pulp tissue or in odontoblasts were cross-

checked to exclude genes with several conflicting probe sets.

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