# Final Report

**Project Title** The response of odontoblasts to exogenous Dentin Matrix Protein 1 (DMP1): its roles in the formation of reactionary dentin

**By Dr. Nattida Charadram**

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# Abstract

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**Project Period :** 2013-2015

**Abstract:** This study provides evidence for the capacity of an organ culture model, based on tooth slices, to disclose the adaptive response of odontoblasts to external stimuli, as a model of the carious process. Short-term cultures of 300 micrometre thick tooth slices were demonstrated to have potential to further probe mechanisms relating to formation of reactionary dentin. Extraction experiments demonstrated that phosphorylated dentin matrix acidic phosphoprotein 1 (DMP1) was mobilised from demineralised dentin in the carious process and detected in reactionary dentin despite profound down-regulation of *DMP1* expression by reactive odontoblasts. It was therefore postulated that during the carious process phosphorylated and non-phosphorylated DMP1 mobilised from the matrix could be recognised by odontoblast receptors thereby contributing to the response typical of reactionary dentin formation. Experimentally, exogenous purified DMP1 induced selective up-regulation of *MMP2* and *TGF-β1* expression with down-regulation of *DSPP*. The regulation of this process was related to translocation of DMP1 within the nuclei of odontoblasts. The down-regulation of *DMP1* mediated by exogenous DMP1 was shown to be largely independent of mediation by an integrin $\alpha v\beta 3$ receptor mechanism. This was interpreted to indicate that DMP1 mobilised within the carious lesion could also modulate odontoblast response patterns.

**บทคัดย่อ :** การศึกษานี้เป็นการศึกษาการตอบสนองของเซลล์สร้างเนื้อฟันของมนุษย์ต่อสิ่งกระตุ้นภายนอกเพื่อศึกษากลไกการตอบสนองต่อรอยโรคฟันผุโดยการสร้างเนื้อฟันปฏิกิริยา (reactionary dentin) โดยใช้ฟันมนุษย์ตัดเป็นแผ่นบางขนาด 300 ไมครอนแล้วนำมาเพาะเลี้ยงในอาหารเลี้ยงเซลล์

จากการศึกษาปริมาณของเดนทีนเมทริกซ์โปรตีนประเภทที่ 1 (dentin matrix protein 1; DMP1) ในฟันผุ พบว่าปริมาณของ DMP1 ที่แยกได้จากเนื้อฟันที่มีการผุนั้นมีปริมาณมากกว่าเนื้อฟันปกติ และยังพบ DMP1 ปริมาณเล็กน้อยในเนื้อฟันปฏิกิริยาซึ่งอยู่ใต้ต่อเนื้อฟันผุ ซึ่งในขณะที่มีการสร้างเนื้อฟันปฏิกิริยานั้นพบว่าเซลล์สร้างเนื้อฟันมีการสร้างโปรตีน DMP1 ลดลง ดังนั้นจึงมีความเป็นไปได้ที่ DMP1 ที่ฝังอยู่ในเนื้อฟันเมื่อเกิดการละลายของแร่ธาตุจากกระบวนการเกิดโรคฟันผุจะถูกปลดปล่อยผ่านมาทางท่อเนื้อฟันทั้งในรูปแบบที่เกิดและไม่เกิดปฏิกิริยาฟอสโฟรีเลชั่น และมากระตุ้นเซลล์สร้างเนื้อฟันให้หยุดยั้งการสร้าง DMP1 และเริ่มสร้างเนื้อฟันปฏิกิริยาขึ้นมา

จากการศึกษาการตอบสนองของเซลล์สร้างเนื้อฟันในแผ่นฟันที่ถูกเลี้ยงในอาหารเลี้ยงเซลล์ที่มีการผสมโปรตีน DMP1 ลงไปพบว่า มีการเปลี่ยนแปลงระดับยีน โดยมีการเพิ่มขึ้นของยีนเมทริกซ์เมทาโลโปรตีนเนส 2 (matrix metalloproteinase 2; MMP-2) และทรานสฟอร์มมิ่ง โกรว์ท แฟคเตอร์-เบต้า (transforming growth factor-beta; TGF-ß) นอกจากนี้ยังพบการลดลงของยีนเดนทีนไซอาโลฟอสโฟโปรตีน (dentin sialophosphoprotein; DSPP) และ DMP1 ซึ่งเป็นการเปลี่ยนแปลงที่คล้ายกับระยะที่เซลล์สร้างเนื้อฟันมีการสร้างเนื้อฟันปฏิกิริยาเพื่อตอบสนองต่อฟันผุ โดยการเปลี่ยนแปลงระดับยีนนี้อาจมีความสอดคล้องกับการเปลี่ยนแปลงตำแหน่งของโปรตีน DMP1 ในนิวเคลียสของเซลล์สร้างเนื้อฟันในแผ่นฟันที่ทำการกระตุ้นด้วยโปรตีน DMP1 ซึ่งมีลักษณะคล้ายกับฟันผุตามธรรมชาติ โดยการเปลี่ยนแปลงของ DMP1 ในเซลล์สร้างเนื้อฟันนี้พบว่าไม่สัมพันธ์กับตัวรับอินทิกริน อัลฟาวีเบตา3 (integrin $\alpha v\beta 3$) ที่อยู่บนผิวเซลล์สร้างเนื้อฟัน

โดยสรุปจากการศึกษานี้พบว่าโปรตีน DMP1 ที่ถูกปลดปล่อยจากเนื้อฟันผุสามารถควบคุมการตอบสนองของเซลล์สร้างเนื้อฟันได้

**Keywords :** reactionary dentin, DMP1, caries, GRP78, $\alpha v\beta 3$

# Executive summary

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There is consensus that progression of the carious process occurs by demineralisation of inorganic matrices of enamel and dentin. Demineralisation by bacterial acids credibly mobilises non-collagenous proteins (NCPs) embedded in dentin. These mobilised exogenous NCPs potentially stimulate the adaptive responses of odontoblasts. The cleaved fragments of DMP1 secreted by odontoblasts during the regulation of dentinogenesis remain in mineralised dentin. DMP1 is able to regulate mineral formation while also functioning as a transcriptional factor to regulate several genes involved in biomineralisation. Dentinogenesis occurs throughout life and is stimulated by external stimuli.

Polymicrobial invasion of dentin in dental caries triggers responses by odontoblasts. A major feature of the adaptive response by odontoblasts is the patterned synthesis of modified dentinal matrix referred to as reactionary dentin, a relatively irregular structure with fewer tubules than physiological dentin. The gene encoding DMP1 is down-regulated during reactionary dentin formation relative to the physiological condition. The absence of DMP1 synthesis during reactionary dentin formation is important for the altered dentinal structure observed in this type of matrix. Reactionary dentin is considered as a biological barrier to protect odontoblasts from invading bacteria. Mobilised exogenous DMP1 released from demineralised dentin could, however, modulate gene expression by odontoblasts during reactionary dentin formation.

This study provides highlight the critical role of exogenous DMP1 during the carious process and reactionary dentin formation.

## Objective

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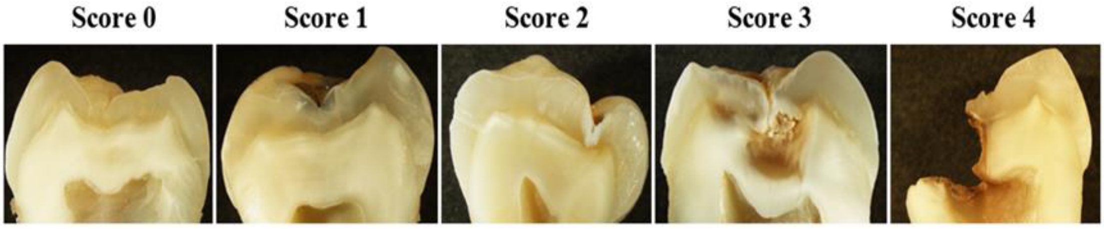
The aim of this study was to explore the possible role of exogenous DMP1 released from demineralised carious dentin in the adaptive response of human odontoblasts to microbial invasion of dentin. Testing of the hypothesis was performed using a short-term tooth slice culture model to preserve the complexity of the tissue.

# Research methodology

Healthy non-carious (*n*=18) and carious (*n*=23) permanent molar teeth were collected from male and female patients, aged 20-35 years, who attended the clinics at the Westmead Centre for Oral health, Westmead Hospital, Australia and dental hospital, Naresuan University, Thailand. The samples were collected from patients who were healthy or presented no systemic conditions known to affect calcified tissues or immune responses. The selected healthy teeth were restoration-free without any signs of attrition, abrasion or erosion leading to dentinal exposure or any related pathologies affecting the pulp-dentin complex. Carious teeth used in this study were restoration-free with coronal caries. Carious teeth were divided into four scores (1, 2, 3 and 4) according to the depth of carious lesion. Score 1 defined carious lesion less than outer half of enamel. Score 2 represented carious lesion invaded not more than one third of dentin. Score 3 and 4 were carious teeth that decay limited within two third of dentin and decay proliferated more than two third of dentin respectively. Healthy teeth were assigned to score 0 (Figure 1).



The Ethics committee of Sydney West Area Health Service and Naresuan University approved the study. The purpose and nature of the research project was explained to patients in writing and a consent form was signed. The decision to extract teeth was made by consent following advice from a clinician who was independent from the study.

### *Fluorescence immunohistochemistry*

Immediately after extraction, the teeth (*n*=5 for each healthy and carious group) were cleaned and a longitudinal groove prepared using diamond bur (NB Nova; Italy) with ample water cooling and without penetration into the dental pulp. Subsequently, the tooth was split into halves by mechanical leverage. The tooth half containing the pulp was fixed in 2% paraformaldehyde/5% sucrose in 0.02 M phosphate buffer, pH 7.4 (680mOsm) for 2 h at 4 °C. Photographs of fixed split tooth halves were taken under a dissecting stereomicroscope (LeicaMZ8; Germany) to confirm proportional representation of the lesion. For teeth that met this additional criterion the half not containing pulp was immediately used for sampling of dentin layers. The fixed tooth half containing pulp was de-mineralised in Morse's solution (22.5% formic acid and 10% sodium citrate) for 3 days at 4 °C, then dehydrated through a graded series of ethanols, 15 min each at 4 °C. A commercial glycolmethacrylate embedding kit (Technovit 8100; Kulzer, Germany) was used for embedding according to the manufacturer's protocol. Tissue blocks were stored tightly sealed at 4 °C until the time of microtomy.

Semi-thin 4 μm resin sections were used for immunohistochemistry. For antigen retrieval, sections were incubated in 1 mM EDTA, pH 8.00 at 100 °C for 30 min in a microwave oven, then sections were cooled to room temperature and washed in PBS for 5 min. Sections were blocked with 10% goat serum/PBS for 3 h followed by incubation overnight with primary antibody diluted in 10% fetal calf serum/PBS at 4 °C. Primary antibodies included: mouse monoclonal DMP1 (dilution 1/200; Invitrogen, Zymed Laboratories), rabbit polyclonal *αvβ*3 (dilution 1/200; Abbiotec, San Diego) and mouse monoclonal glucose-regulated protein 78 (GRP78) (dilution 1/200; Invitrogen, Zymed Laboratories). Negative control sections were also incubated overnight with isotype control antibody. After washing in PBS (3×10 min), sections were incubated with fluorochrome-conjugated secondary antibody diluted in 10% fetal calf serum/PBS at room temperature for 2 h. The secondary antibodies were goat anti-mouse IgG Alexa-594 (dilution

1/500; Invitrogen, Zymed Laboratories) or goat anti-rabbit IgG Alexa-488 (dilution 1/500; Invitrogen, Zymed Laboratories). The sections were then washed in PBS and mounted onto glass slices using ProLong Gold antifade reagent with DAPI (Invitrogen, Molecular Probes; Eugene, OR). The sections were viewed using an Olympus BX60 fluorescence microscope and images captured using a Leica DFC500 camera.

### *Extraction of dentin proteins*

Dentin proteins from healthy and carious teeth which decay did not extend more than 2/3 of dentin (*n*=5 for each healthy and carious group) were extracted as describe previously [33]. Three different dentin layers from carious teeth were collected using a round steel bur (Premier; USA). The first layer was collected from the soft carious lesion, the second layer comprised caries-affected dentin, and finally the third layer was the reactionary dentin area (Figure 1A). Sound dentin was collected from the crowns of healthy teeth. Pulverised dentin was demineralised in 10% EDTA (pH 7.4) with addition of proteinase inhibitors (2.5 mM benzamidine HCl, 50 mM ε-amino-n-caproic acid, 0.5 mM N-ethyl maleimide, and 0.3 mM phenyl methyl sulphonyl fluoride) for 24 h at 4°C under constant agitation. The first supernatant EDTA (E1) was collected by centrifugation at 2000 g for 10 min and stored at -20°C. Demineralised dentin was extracted in 4 M guanidine HCl in 0.05 M TRIS pH 7.4 with proteinase inhibitors for 48 h at 4 °C under constant agitation. The first guanidinium chloride extract (G1) was collected by centrifugation at 2000 g for 10 min and stored at −20 °C. After guanidinium extraction, dentin samples were demineralised in 10% EDTA (pH 7.4) with proteinase inhibitors for 24 h under the same conditions. The second supernatant EDTA (E2) was collected and stored at−20 °C. Dentin samples were suspended in 4 M guanidine HCl in 0.05 M TRIS pH 7.4 with proteinase inhibitors for 48 h under the same conditions and the second guanidinium chloride extracts (G2) collected and stored at−20 °C. The combination of supernatant EDTA and guanidinium chloride extract (E1+G1+E2+G2) was dialysed by using the 10 kDa nominal molecular-weight cut-off (MWCO)

Slice-A-Lyzer dialysis cassette (Thermo Scientific; USA) against a large volume of distilled water changed every 12 h for 3 days at 4°C and then concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore; USA). Total protein yield from each sample was measured spectrophotometrically by Bradford protein assay (Thermo Scientific; USA).

### *Western immunoblot of DMP1 and determination of its phosphorylation status in extracts from human dentin*

Total dentin protein (10 μg) from each sample was fractionated on 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, (0.45 μm; BIO-RAD, Germany) at 350 V, 300 mA constant current for 2 h at 4°C. Total dentin protein was resolved on SDS-PAGE and stained with Coomassie blue. The blots were blocked with 0.5% bovine serum albumin/ PBS overnight followed by incubation with primary antibody-mouse monoclonal anti-DMP1 (dilution 1:500; Invitrogen, Zymed Laboratories) for 3 h at room temperature. After extensive washing with TBST (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20), blots were incubated with secondary antibody-polyclonal goat anti-mouse immunoglobulin/ AP (dilution 1:500; Dako; Glostrup; Denmark) for 2 h. The blots were washed with TBST and developed using alkaline phosphatase-conjugated substrate kit (BIO-RAD; Germany).

To investigate the phosphorylation of DMP1 extracted from human dentin, the proteins were transferred on to nitrocellulose membrane and blocked as described above. The blots were then incubated with primary antibody mouse monoclonal anti-phosphoserine (dilution 1:500; Invitrogen, Zymed Laboratories) for 3 h at room temperature. The blots were washed, incubated in secondary antibody and stained as described above.

### *Immuno-gold labelling of DMP1*

Immuno-gold labelling for DMP1 was performed as described previously [33]. Immediately after extraction, healthy and carious teeth which decay did not extend more than 2/3 of dentin ($n$=3 for each group) were cleaned and serial sections of 500 µm thickness longitudinal sections of tooth crown were prepared using a water-cooled slow-speed diamond saw sectioning machine (Isomet 5000, Buehler Ltd., Lake Bluff, IL, USA). The sections were pre-fixed in 4% paraformaldehyde solution at 4°C for 12 h, and then rinsed with PBS. Samples were immersed in 0.05 M TRIS HCL buffer solution (TBS) at pH 7.6 with 0.15 M NaCl and 0.1% bovine serum albumin, followed by 3 rinses for 10 min each. Samples were blocked in 10% goat serum in 0.05 M TBS at pH 7.6 for 30 min at room temperature, followed by incubation overnight at 4 °C with primary antibody, mouse monoclonal DMP1 (dilution 1/100; Invitrogen, Zymed Laboratories), diluted in 0.05 M TBS, pH 7.6. Negative control sections were also incubated overnight with isotype control antibody. After rinsing with 0.05 M TBS at pH 7.6 and 0.02 M TBS at pH 8.2, samples were incubated with secondary antibody, goat anti-mouse IgG conjugated with 20 nm colloidal gold particles (diluted 1:20; BBI international, UK) in 0.02 M TBS at pH 8.2 for 90 min at room temperature and rinsed with 0.02 M TBS, pH 8.2. The samples were post-fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2 for 4 h and rinsed with 0.1M cacodylate buffer at pH 7.2 for 1 h. After dehydration in a graded ethanol series (30%, 50%, 70%, 90%, 95% and 3 changes of 100%), samples were dried using hexamethyl disilazane (Sigma-Aldrich; St Louis, MO, USA) and mounted on stubs using a conductive tape and coated with gold/palladium (Emitech K550x; UK). Observations were performed using high resolution field emission gun scanning electron microscopy, FEG-SEM (Ultra Plus; Carl Zeiss, Germany). Images were obtained using a combination of Secondary Electron (SE2) and Energy selected Backscattered (EsB) detectors at 10.00 kV acceleration voltages.

### *Tooth slice cultures*

Freshly extracted sound third molars (*n*=10) were kept in cold sterile Dulbecco's modified Eagle medium/ Nutrient Mixture F-12 (Ham) (Gibco; Invitrogen, Zymed Laboratories) supplemented with antibiotic-antimycotic solution (Gibco; Invitrogen, Zymed Laboratories), with a final concentration of 100 I.U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, 10% (v/v) fetal bovine serum (FBS), 1 µg/ml vitamin K1 (Sigma-Aldrich; St Louis, MO, USA), 50 µg/ml vitamin C (Sigma-Aldrich; St Louis, MO, USA) until processed for tooth slice cultures. The teeth were rinsed with 5% NaOCl then sterile PBS. The plaque, calculus and periodontal tissues were removed followed by a rinse with 70% ETOH then sterile PBS. Serial longitudinal sections of tooth of 300 µm thickness were prepared using a water-cooled slow-speed diamond saw sectioning machine (Isomet 5000, Buehler Ltd., Lake Bluff, IL, USA).

One of the tooth slices that contained pulp was immediately fixed in 2% paraformaldehyde/5% sucrose in 0.02 M phosphate buffer, pH 7.4 (680mOsm) for 2 h at 4 °C followed by de-mineralisation in Morse's solution (22.5% formic acid and 10% sodium citrate) for 3 days at 4 °C. Samples were then dehydrated and embedded in resin as described previously and used as an internal control. The other tooth slices that contained pulp were rinsed with medium then transferred to the upper chamber of 24 mm transwells with 3 µm pore polyester membrane inserts (Costar; Corning Inc., NY, USA). The medium was filled to cover the bottom half of slice (Figure 3). Cultures were incubated at 37°C in a 5% $CO_2$ atmosphere.

### *Lactate dehydrogenase (LDH) assay*

The vitality of organ cultures was monitored by detecting LDH released from dying cells. The assay was performed using the In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma-Aldrich; St Louis, MO, USA) as suggested by manufacturer. The medium from the cultures were collected for the assay every 30 min for the first 6 h, then at 18, 20, 22, 24, 48, 72, 96, 122 and 144 h. The absorbances were measured spectrophotometrically at a wavelength of 490 nm and the background absorbances were subtracted from all readings. The line graph was plotted as a percentage of maximum LDH release.

### *Purity and phosphorylation status of human recombinant DMP1*

A commercial recombinant human DMP1 (4129-DM; R&D systems) secreted by an engineered cell line was used for this study. As there was no supportive data available for the specific purity and the phosphorylation status of this product, these parameters were evaluated.

### 2.7.1 Western immunoblot of DMP1 and phosphoserine

Recombinant human DMP1 (4129-DM; R&D systems) (2 µg) was fractionated on 10% SDS-PAGE. The protein was transferred on to a nitrocellulose membrane and blocked as described above. The blots were then incubated with primary antibody, mouse monoclonal anti-DMP1 (dilution 1:500; Invitrogen, Zymed Laboratories ) and mouse monoclonal anti-phosphoserine (dilution 1:500; Invitrogen, Zymed Laboratories) for 3 h at room temperature. The blots were washed, incubated with secondary antibody and stained as described above.

### 2.7.2 Mass spectrometry (MS)

Protein preparation for mass spectrometry (MS) was based on methods described previously [36]. Briefly, 20 µl of human recombinant DMP1 was fractionated on 10% SDS-PAGE. The two prominent protein bands were dissected and digested in 25 mM $NH_4HCO_3$ containing 12.5ng/ µl trypsin in a final volume of 30 µl for 16 h at $37^0$C. Peptides were desalted and concentrated by $C_{18}$ Zip-Tips (Millipore) and eluted in 70% acetonitrile with 0.1% TFA. Extracted peptides were overlayed onto 0.5 µl of α-cyano-4-hydroxycinnamic acid (5mg $ml^{-1}$ in 70% acetonitrile/0.1% TFA) for MS analysis. MALDI-time-of flight (TOF) and MS/MS was performed on the AB SCIEX TOF/TOF™ 5800 System.

### *Extracellular treatment with recombinant human DMP1*

To study the effects of extracellular DMP1 on odontoblasts in the tooth slice culture, recombinant human DMP1 was added to the medium at a concentration of 250 ng/ml. The tooth slices were then incubated at 37°C in a 5% $CO_2$ atmosphere for 1 h 30 min. After incubation, tooth slices were fixed in 2% paraformaldehyde/5% sucrose in 0.02 M phosphate buffer, pH 7.4 (680mOsm) for 2 h at 4 °C, followed by de-mineralisation in Morse's solution (22.5% formic acid and 10% sodium citrate) for 3 days at 4 °C. Specimens were dehydrated and embedded in resin as described above. Tissue blocks were stored tightly sealed at 4 °C until the time of microtomy.

### *Blockade of αvβ3 integrin receptors in odontoblasts in tooth slice cultures*

To investigate the importance of *α*v*β*3integrin receptor for a putative DMP1 functional pathway in odontoblasts in the tooth slice cultures, odontoblast *α*v*β*3 integrin receptors were blocked by incubation in 20 µg/ml of a mouse anti-human integrin *α*v*β*3 monoclonal antibody demonstrated to be a effective in blockade [24] (Millipore; USA) for 1 h 30 min at 37°C in a 5% $CO_2$ atmosphere followed by incubation with 250 ng/ml rDMP1 for 1 h 30 min at 37°C in a 5% $CO_2$ atmosphere. The negative control was incubated with isotype control antibody prior to the addition of rDMP1. After incubation, tooth slices were fixed in 2% paraformaldehyde/5% sucrose in 0.02 M phosphate buffer, pH 7.4 (680mOsm) for 2 h at 4 °C followed by de-mineralisation in Morse's solution (22.5% formic acid and 10% sodium citrate) for 3 days at 4 °C. Slices were dehydrated and embedded in resin as described above. Tissue blocks were stored tightly sealed at 4 °C until the time of microtomy.

### *Gene expression in odontoblasts of tooth slice cultures*

The investigation of the change in expression of genes of interest was performed as described previously [33]. Briefly, 12 consecutive 8 µm sections of resin-embedded odontoblast layers were micro-dissected from tooth crowns under a stereomicroscope (Leica Microsystems; Germany) using microsurgical knives (Unique Technologies Inc; Mohnton, USA).

RNeasy FFPE kit (Qiagen) was used to extract total RNA from the sections according to the manufacturer's instruction. Total RNA yields from the extraction were quantified by A260 nm measurement (Nanodrop ND-1000; Thermo Scientific) and quality of RNA in samples was investigated by Agilent RNA 6000 Pico chip (Agilent Technologies; Palo Alto, CA). Reverse transcription of extracted total RNA was performed using SuperScript II reverse transcriptase (Invitrogen; Zymed Laboratory) according to the manufacturer's protocol. Briefly, the reaction contained 4 µl of RNA, 1 µl of SuperScript II reverse transcriptase, 2 µl of random nanomer (Geneworks; Adelaide, South Australia) (final concentration of 10 µmol/L), 1 µl of deoxyribonucleotide triphosphate mix (10 mM each), 4 µl of 5X first-strand buffer, 2 µl of 0.1 M dithiothreitol, 1 µl of RNase inhibitor (40 units/reaction) and 5 µl of DEPC-treated water. The reverse transcription product, cDNA,was stored at−20 °C until analysis. Singleplex real-time qPCR was performed by using 2 µl of cDNA of the genes of interest for assay on a Strategene Mx3005P Real-Time PCR system (Agilent Technologies; Palo Alto, CA) with the Platinum quantitative PCR Supermix-UDG (Invitrogen; Zymed Laboratories). Primers and Taqman probes were designed to span exon-exon junctions to prevent genomic DNA amplification for DMP1, dentin sialophosphoprotein (DSPP), matrix metalloproteinase 2 (MMP2), tissue inhibitor of metalloproteinase 2 (TIMP2), membrane type 1- matrix metalloproteinase (MT1-MMP), Toll-like receptors 2 and 4 (TLR2, 4), transforming growth factor - β1 (TGF-$\beta$1), collagen type Iα1 (COL1A1), collagen type Iα2 (COL1A2) and hypoxia inducible factor - 1 α (HIF-1α) (Table 1).

## Table 1

Taqman primer and probe sets

| Gene | GeneBank Accession no. | Oligos | Primers (5'-3') |
|---|---|---|---|
| *DMP1* | NM_004407 | F-primer | TGT GAA CTA CGG AGG GTA GAG G |
|  | NM_004407 | R-primer | ACT GGG AGA GCA CAG GAT AAT CC |
|  |  | Probe | CAC ACC CAA CTA TGA AGA TCA GCA TCC TGC TCA TGT TCC T |
| *DSPP* | NM_014208 | F-primer | GCA GAA GGA TAG AGA AAG CAA ACG |
|  |  | R-primer | GGG ACC CTT GAT TTC TAT TCC CTT ATC |
|  |  | Probe | CCA AAG AAT CAG AGA CAC ATG CTG TTGG GAA GAG CC |
| *TGF-β1* | NM_011577 | F-primer | CTA CCA TAG CCA ACT TCT GCC TC |
|  |  | R-primer | GCC AGG ACC TTG CTG TAC T |
|  |  | Probe | CCC TGC CCC TAC ATT TGG AGC CTG GAC A |
| *MMP2* | NM_004530.4 | F-primer | CGG CGG TCA CAG CTA CTT CTT C |
|  |  | R-primer | CCC TGG CTC CCA CAG GCC CTT CCT |
|  |  | Probe | GTT CTC CAG GCC CGG TGT AT |
| *TIMP2* | NM_003255.4 | F-primer | GGC TGC GAG TGC AAG ATC AC |
|  |  | R-primer | CGT GCT ACA TCT CCT CCC CGG ACG AGT GCC T |
|  |  | Probe | CCT GGT GCC CGT TGA TGT TCT TC |
| *MT1-MMP* | NM_004995.2 | F-primer | CTGTCAGGAATGAGGATCTGAATGGA |
|  |  | R-primer | TGC ACG AGC TGG GCC ATG CCC TG |
|  |  | Probe | CACTGGTAAAAGGGTGCCATGAT |
| *TLR2* | NM_003264.3 | F-primer | TGGTAGTTGTGGGTTGAAGCAC |
|  |  | R-primer | TCCTTGGAGAGGCTGATGATGACCCCCAAGACC |
|  |  | Probe | ACAGAGAAGCCTGATTGGAGGAT |
| *TLR4* | NM_138554.3 | F-primer | GGACTCTGATCCCAGCCATG |
|  |  | R-primer | AGC CTC TCT CCA GGT GCC CAT CAG AAG CG |
|  |  | Probe | GGGAACTCTTTCTTCATTGCCTTGTAC |
| *COL1A1* | NM_000088.3 | F-primer | GAAC AAG GTC CCT CTG GAG C |
|  |  | R-primer | TGC TGG TCC CCG AGG TCC CCC |
|  |  | Probe | GTT GAG TCC ATC TTT GCC AGG A |
| *COL1A2* | NM_000089.3 | F-primer | GGT CCT ATT GGA AGC CGA GG |
|  |  | R-primer | CCA GGT TCA CCC TTG TTT CCA TCA GGC CCT |
|  |  | Probe | CAG TGC CCA CAG CAC CAA |
| *HIF1A* | NM_ 001530.3 | F-primer | GCC GCT GGA GAC ACA ATC ATA TC |
|  |  | R-primer | TCA AGT TGC TGG TCA TCA GTT TCT GTG TCG TTG CTG CC |
|  |  | Probe | GAT GTA ATG CTC CCC TCA CCC AA |
| *ACTB* | NM_001101 | F-primer | CCT GAC GGC CAG GTC ATC AC |
|  |  | R-primer | GAC TCC ATG CCC AGG AAG GA |
|  |  | Probe | CCG CTG CCC TGA GGC ACT CTT CCA G |

Each sample was run in triplicate in a 25 μl reaction volume on a 96-well PCR plate. The housekeeping gene beta-actin (ACTB) was used as the most constant endogenous reference control [35]. Raw data was analysed and exported using MxPro QPCR software. Raw fluorescence data was imported to an automated calculation workbook entitled Data Analysis for Real-Time PCR (DART-PCR) which enables rapid calculation of threshold cycles, amplification efficiency and resulting R0 values (together with the associated error) [37]. Results for each gene were normalized against *ACTB* to obtain the fold change in expression value. Eight microlitres of PCR amplification product of each gene was resolved on 2% agarose gel and stained with GelRed (Biotium) compared to *ACTB.*

### ***Statistical analysis***

SPSS statistical software (SPSS v.16; Chicago, Illinois, US) was used for the statistical analysis of data. All data are presented as mean ± SD. Statistical significance was determined using a two-tailed Student's t-test assuming equal variance. In the present study, a P-value $\leq 0.05$ was considered as statistically significant.

# Result

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### *Toluidine blue stain of dentin and odontoblasts from healthy and different stages of carious teeth*

Healthy teeth showed intact odontoblast layer without the presence of reactionary dentin as well as stage 1 carious teeth. Reactionary dentin was observed from carious stage 2 with less number of dentinal tubules compared to physiological dentin. Reduction of odontoblast cells was noticed under the area of reactionary dentin formation. Stage 4 carious teeth revealed the disrupted dental pulp morphology. (Figure 2)

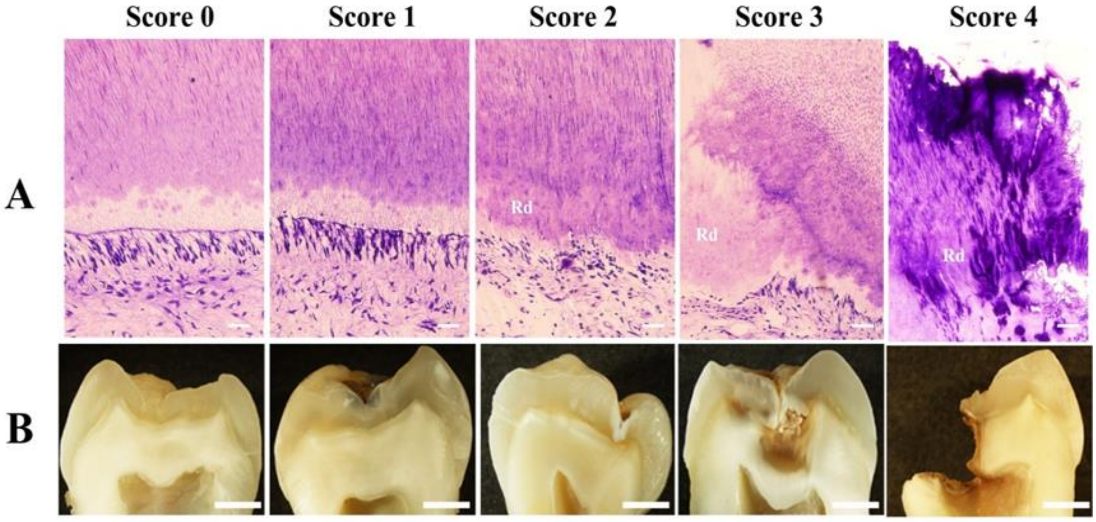


**Figure 2: Toluidine blue stain of dentin and odontoblasts from healthy and different stages of carious teeth.** (A) Images of toluidine blue staining (score 0 = healthy tooth, score 1-4 = different stages of carious teeth). Reactionary dentin (Rd) was seen from stage 2 carious teeth. Intact odontoblast layer was observed in healthy tooth (stage 0) and early enamel carious lesion (stage 1). Necrosis of dental pulp with severe destruction of dentin was observed in late stage of carious teeth (score 4) (Scale bar = 50 µm) (B) Longitudinal section of healthy (score 0), early enamel carious lesion (score 1), carious lesion extend ≤ 1/3 of dentin (score 2), carious lesion extend ≤ 2/3 of dentin (score 3) and score 4 showed advance carious lesion proliferate ≥ 2/3 of dentin. (Scale bar = 5 mm)

### *Western immunoblot and phosphorylation status of DMP1 in different carious layers*

Extracted dentin proteins from healthy and carious teeth contain a COOH-terminal fragment of DMP1 (DMP1-C) as a 57-kDa fragment (Figure 3A). Similar amounts of DMP1-C were evident in sound dentin and affected physiological dentin (Pd) (Figure 3A). An intense band of DMP1-C was observed in extracts of soft carious lesions. In reactionary dentin (Rd), only trace amounts of DMP1-C could be extracted (Figure 3A). The full-length form and $NH_2$-terminal fragment of DMP1 (DMP1-N) were not detected in extracts of dentin.

Serine residues contribute the majority of phosphorylation sites on DMP1 (George et al., 1993; Qin et al., 2003). Bands representing peptides containing phosphorylated serine were present in dentin extracts indicating the phosphorylated form of DMP1-C. However, the band representing phosphorylation of DMP1 from carious dentin was less intense than total DMP1 detected in this layer (Figure 3A).

### *Immuno-gold labelling of DMP1by ultra-high resolution FEG-SEM*

The localisation of DMP1 was evaluated by immuno-gold labelling. DMP1 was localised on partially demineralised dentin surfaces of healthy and carious teeth under ultra-high resolution FEG-SEM. A combination of SE2 and EsB was applied to simultaneously reveal immuno-gold labelling and related substrate morphology. Positive labelling was indicated by white spherical spots. DMP1 was localised around dentinal tubules in healthy teeth and affected dentin in carious teeth. In reactionary dentin from carious teeth, labelling for DMP1 was not detected (Figure 3B).

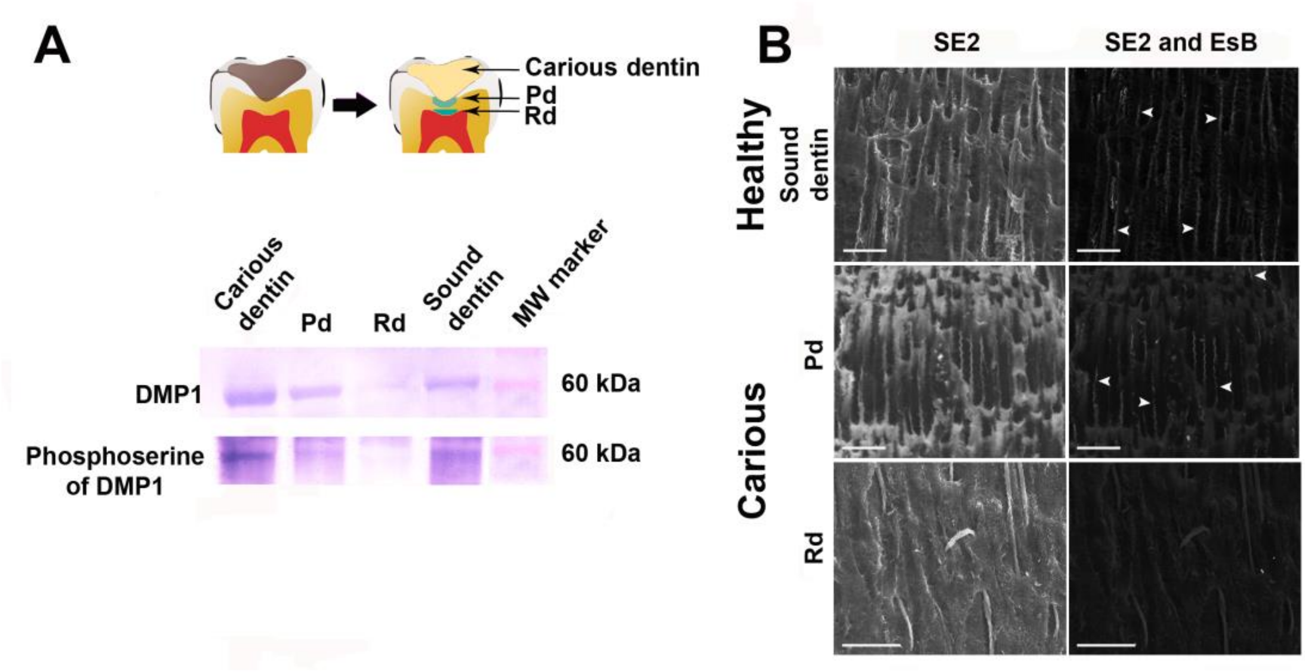


**Figure 3: Western immunoblot of DMP1 and phosphoserine of dentin proteins extracted from different layers of carious dentin and immuno-gold labelling of DMP1 from healthy and carious samples.** (A) Diagram of dentin layers: carious dentin, physiological dentin (Pd) and reactionary dentin (Rd). Immuno-labelling of DMP1-C at ~57 kDa is more intense from dentin proteins extracted from carious dentin compared to physiological dentin (Pd), reactionary dentin (Rd) and sound dentin from healthy teeth. Immuno-labelling for phosphoserine shows a band at ~57 kDa. The intensity of phosphorylated serine of DMP1 from the carious sample was relatively lower than for immuno-labelling of DMP1 peptide. (B) Immuno-gold labelling of DMP1 shows the accumulation of DMP1 in peri-tubular dentin of sound dentin from healthy samples and physiological dentin from carious samples. The absence of DMP1 in reactionary dentin is apparent. Scale bar 10 µm.

### *Immunofluorescence of DMP1 in odontoblasts from healthy and different stages of carious teeth*

DMP1 signaling was intense in odontoblasts obtained from healthy (score 0) and early enamel carious teeth (score 1). Positive labeling located in both nucleus and cytoplasm. The amount of DMP1 significantly decreased in carious samples score 2 and 3. Late stage carious teeth (score 4) was not use in this study according to the necrosis of dental pulp. (Figure 4)

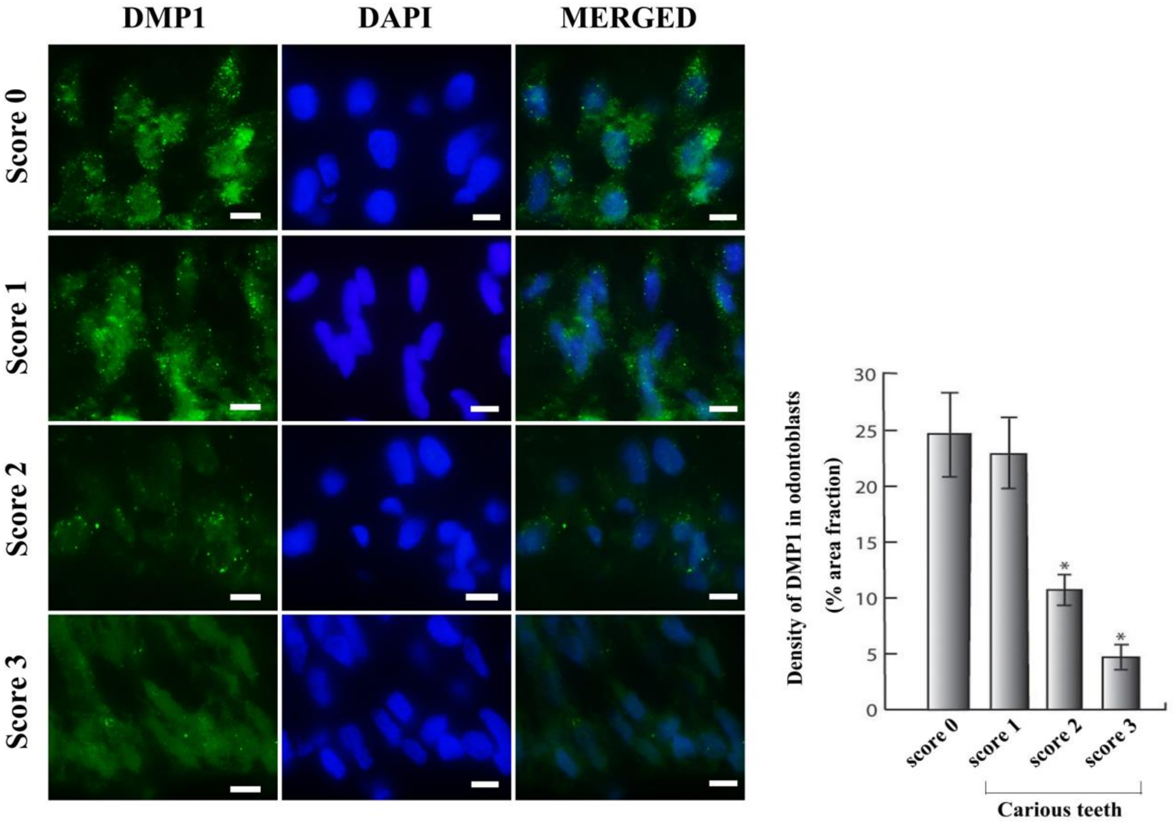


**Figure 4: Immunofluorescence of DMP1 in odontoblasts from healthy and different stages of carious teeth.** DMP1 signaling presents in bright green fluorescence (row 1), nuclei staining with DAPI was demonstrated in blue (row 2), and row 3 shows merged image of DMP1 labeling with DAPI. Positive labeling of DMP1 was intense in nucleus and cytoplasm of healthy and carious teeth score 1. Significantly reduction of DMP1 was observed in stage 2 and 3 carious teeth. Scale bar 10 µm.

### *Immunofluorescence of DMP1, αvβ3 integrin receptor and GRP78 in odontoblasts from healthy and carious teeth*

Positive labelling of DMP1 was indicated by fluorescence restricted to odontoblasts (Figure 5). Staining for integrin $\alpha v\beta 3$ receptor was more intense in the odontoblast layer from carious teeth relative to healthy teeth (Figure 5). In contrast, staining for DMP1 was more intense in the odontoblast layer from healthy teeth compared to carious teeth (Figure 5). Integrin $\alpha v\beta 3$

receptors were localised on the cell surface while DMP1 was distributed in both cytoplasmic and nuclear compartments (Figure 5).

The immuno-labelling of DMP1 in dental pulp shows intense reaction in odontoblasts and their nuclei but not the other cells (see also Figure 11). Observation of the distribution pattern of DMP1 in odontoblasts indicated 4 patterns of distribution (see also Figure 12);

(a) uniformly distributed inside the nucleus with additional cytoplasmic staining,
(b) uniform distribution inside the nucleus without cytoplasmic staining,
(c) patterned nuclear staining with cytoplasmic reaction,
(d) patterned nuclear staining without cytoplasmic staining.

In healthy teeth, all of the distribution patterns were observed (see also Figure 12). In carious teeth the (c) and (d) type patterns were predominant (see also Figure 12).

Glucose-regulated protein 78 (GRP78) is one the chaperone proteins and was reported to mediate endocytosis of DMP1 (Ravindran et al., 2011). Staining for GRP78 was more intense from the cell surface to outer surface of nucleus in odontoblasts from carious compared to healthy teeth (Figure 5).

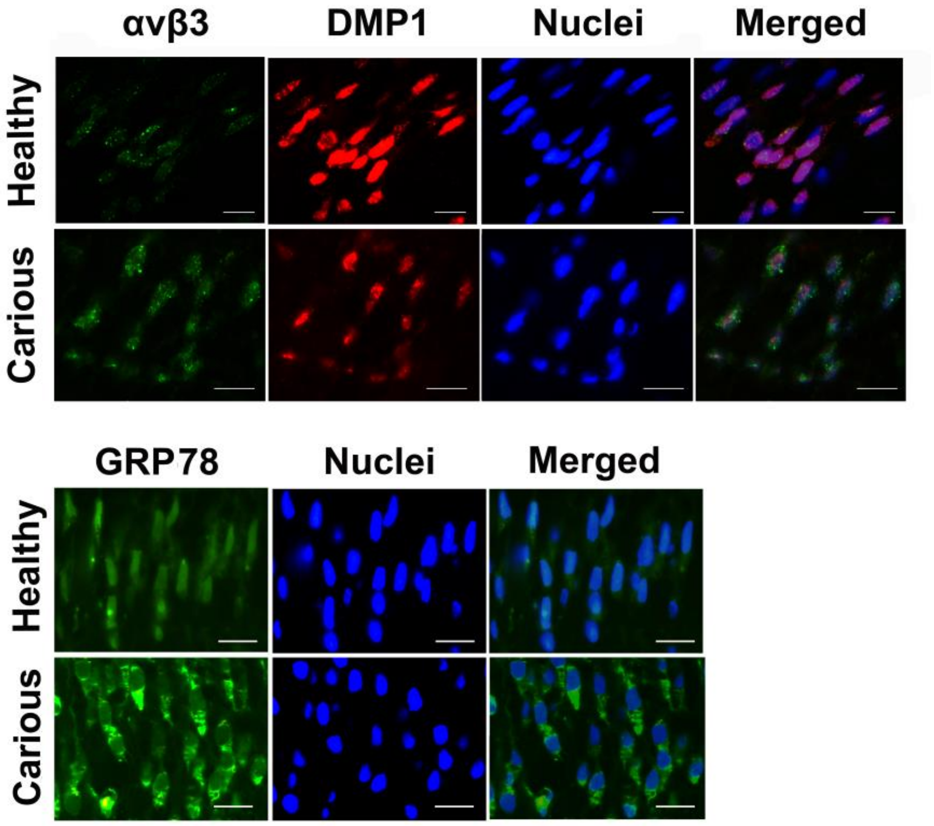


**Figure 5: Immunofluorescence of DMP1, integrin *αvβ*3 and GRP78 in the odontoblast layer.** Odontoblast nuclei were stained with DAPI (third and fourth panels). Sections were dual stained for DMP1 (red fluorescence) and integrin *αvβ*3 (green fluorescence). DMP1 labelling was more intense in the odontoblastic layer from healthy compared to carious teeth (second panel). In contrast, integrin *αvβ*3 was more intense in the odontoblastic layer of carious teeth (first panel). Similarly, GRP78 labelling was more intense in the odontoblastic layer of carious teeth. Scale bar 30 µm.

### *Lactate dehydrogenase (LDH) assay indicating vitality of cultured tissues*

The culture set-up is outlined in Figure 6. LDH assay was performed to investigate the time frame for the best condition to maintain the vitality of cells in the tooth slice culture model. LDH release was $\leq$ 10% of total within the first 6 h (Figure 7B) indicating the good condition of the cells. This was confirmed by Toluidine blue staining of odontoblast layers showing intact odontoblasts (Figure 7A). This provides confidence for this study as the maximum culture time was 3 h (Figure 7B).

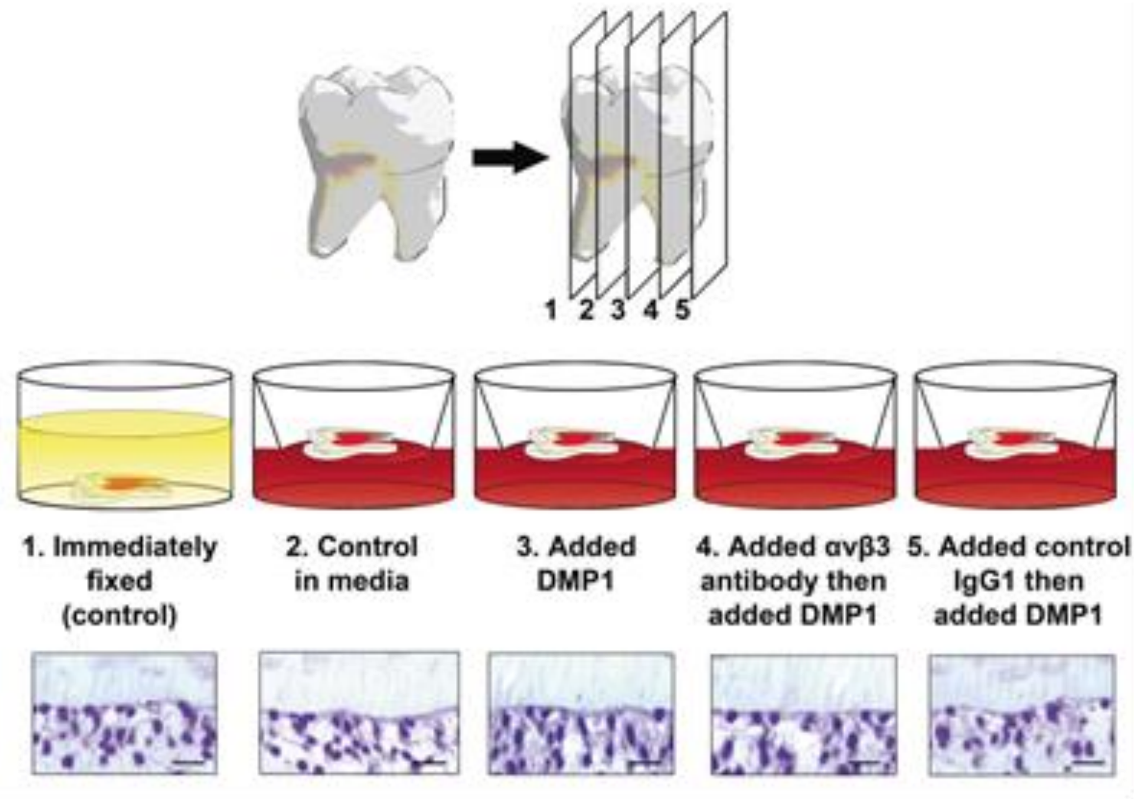


**Figure 6: Diagrammatic representation of the tooth slice culture model.** Healthy teeth were sliced into five slices of 300 µm thickness. The first slice was immediately fixed as an internal control, the second slice was cultured in media without exposure to DMP1 as another internal control, the third slice was cultured in media with added DMP1, the fourth slice was cultured in media and integrin *αvβ*3 was blocked prior to addition of

DMP1 and the fifth slice was cultured in media with isotype control IgG1 prior to addition of DMP1 as a control for anti- integrin *αvβ*3 antibody. Toluidine blue stain of odontoblast layers demonstrated intact odontoblasts after the culture period. Scale bar 30 µm.

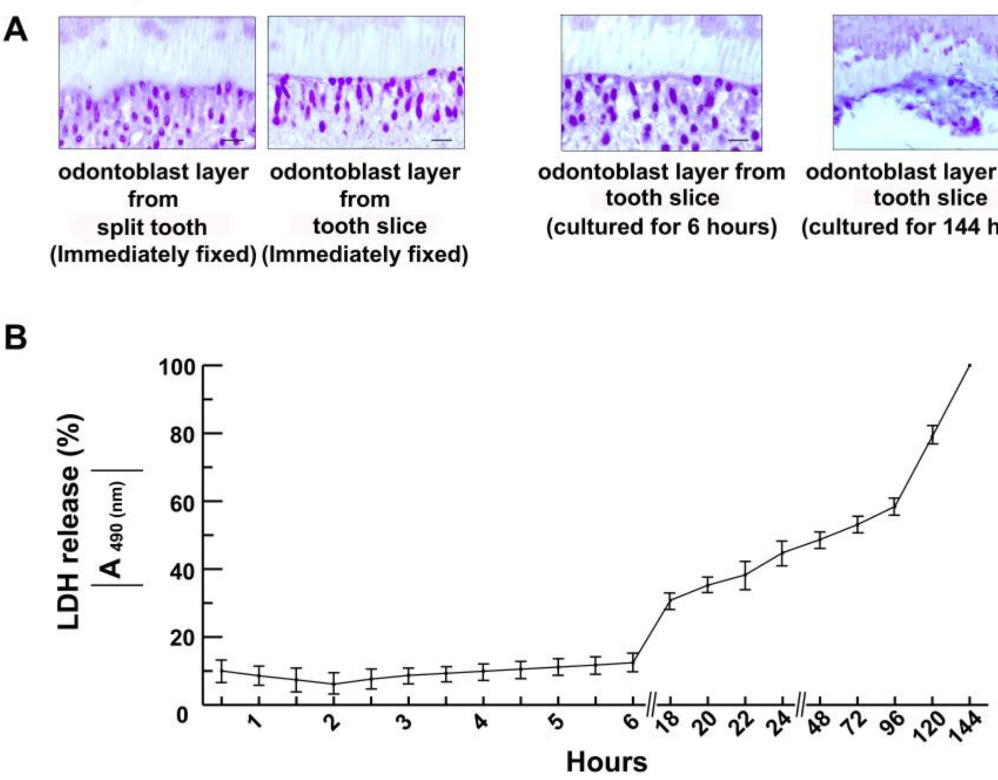


**Figure 7: Line graph representing the vitality of cultured tissue observed over 6 days and Toluidine blue stain of odontoblast layers to demonstrate the physical structure of odontoblasts.** (A) Toluidine blue stain of odontoblast layers from split tooth (first panel) compared to odontoblast layers from tooth slice (second panel). The slice process caused mild damage to odontoblast layer evident by spacing between odontoblasts. However, numbers of intact odontoblasts are observed. (B) Line graph presents LDH release in percentages of total from tooth slice cultures over different times up to 144 h. LDH release was ≤ 10% within the first 6 h and reached maximum release at day six. All values depict mean±SD (*n*=3). (A) Toluidine blue stain of odontoblast layers from 6 h-tooth slice culture shows intact odontoblasts (third panel). Fourth panel displays corrupted odontoblasts at 144 h-tooth slice culture. Scale bar 30 µm.

## *Purity and phosphorylation status of commercial human DMP1*

The apparent purity and phosphorylation of the commercial preparation was evident by Coomassie blue staining of product resolved on 10% SDS-PAGE (Figure 8), Western blot (Figure 8) and MS analysis (Table 2 and 3). The SDS-PAGE presents the strongest band as DMP1-C with the presence of full-length DMP1 and very weak expression of DMP1-N confirmed by Western blot of DMP1 (Figure 8). The MS analysis also confirmed the peptide sequence match with *Homo* *sapiens* DMP1 (data not shown). There were weak separated bands present above all three forms in SDS-PAGE (Figure 8). MS analysis suggested that few possible phosphorylation sites occurred in both full-length and DMP1-C (Table 2 and 3). The phosphoserine of recombinant DMP1 was not detected on Western Blot (data not shown).

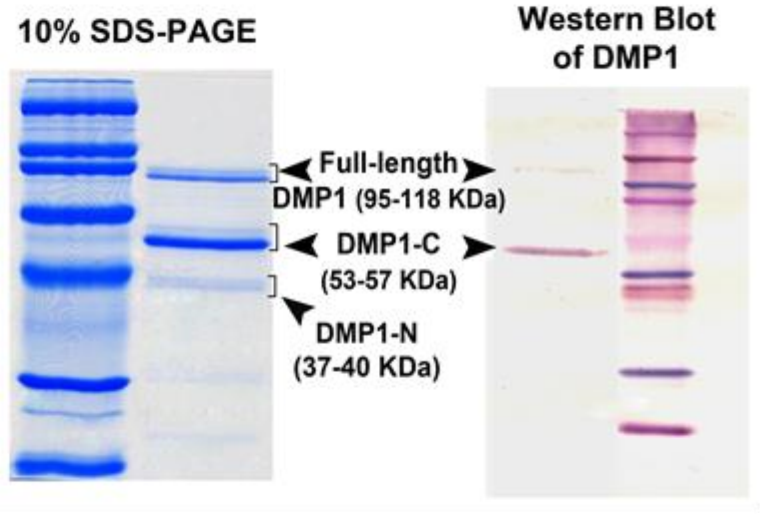


**Figure 8: SDS-PAGE and Western blot of DMP1 from commercial DMP1.** The strongest band for DMP1-C was observed at ~53-57 kDa with the presence of full-length DMP1 at ~95-118 kDa on SDS-PAGE. This was confirmed by Western blot of DMP1.

## Table 2: Mass spectrometry analysis of full length DMP1

Few potential phosphorylation sites were identified on the peptide sequences of full-length DMP1.

| Observed | Mr(expt) | Mr(calc) | Peptide |
|---|---|---|---|
| 860.4083 | 859.4010 | 859.4399 | K.NIEIESR.K |
| 864.4516 | 863.4443 | 863.3985 | R.STGKGGDDK.D |
| 873.4068 | 872.3995 | 872.4352 | R.DTGLSQPR.R |
| 873.4068 | 872.3995 | 872.4352 | R.DTGLSQPR.R |
| 1018.4505 | 1017.4432 | 1017.4588 | K.DRQEGGNSR.L |
| 1079.4652 | 1078.4579 | 1078.4778 | K.SSSEEDGQLK.N |
| 1329.5393 | 1328.5320 | 1328.4291 | R.GNSRMNSAGMK.S + Deamidated (NQ); Oxidation (M); 2 PhosphoSTY (STY) |
| 1657.6401 | 1656.6328 | 1656.6539 | R.YQNNESEDSEEWK.G |
| 1920.8756 | 1919.8683 | 1919.9072 | K.SSSEEDGQLKNIEIESR.K |
| 1920.8756 | 1919.8683 | 1919.9072 | K.SSSEEDGQLKNIEIESR.K |
| 2367.9661 | 2366.9588 | 2367.0020 | R.ISEEDDRSELDDNNTMEEVK.S |
| 2383.9626 | 2382.9553 | 2382.9969 | R.ISEEDDRSELDDNNTMEEVK.S + Oxidation (M) |
| 2657.1201 | 2656.1128 | 2656.1597 | K.ESGENSEQANTQDSGGSQLLEHPSR.K |
| 2657.1201 | 2656.1128 | 2656.1597 | K.ESGENSEQANTQDSGGSQLLEHPSR.K |
| 2679.1294 | 2678.1221 | 2678.0003 | K.LTVDAYHNKPIGDQDDNDCQDGY.- + 3 Deamidated (NQ); PhosphoSTY (STY) |
| 2742.9626 | 2741.9553 | 2741.9809 | K.GGDDKDDDEDDSGDDTFGDDDSGPGPK.D |

| | | | |
|---|---|---|---|
| 2785.2148 | 2784.2075 | 2784.2546 | K.ESGENSEQANTQDSGGSQLLEHPSRK.I |
| 2785.2148 | 2784.2076 | 2784.2546 | K.ESGENSEQANTQDSGGSQLLEHPSRK.I |
| 2872.2498 | 2871.2425 | 2871.2867 | K.SKESGENSEQANTQDSGGSQLLEHPSR.K |
| 2872.2498 | 2871.2425 | 2871.2867 | K.SKESGENSEQANTQDSGGSQLLEHPSR.K |
| 3116.1570 | 3115.1497 | 3115.1217 | K.SKESGENSEQANTQDSGGSQLLEHPSR.K + 4 Deamidated (NQ); 3 PhosphoSTY (STY) |
| 3456.3945 | 3455.3872 | 3455.4204 | R.LGSDEDSDDTIQASEESAPQGQDSAQDTTSESR.E |
| 3456.3945 | 3455.3873 | 3455.4204 | R.LGSDEDSDDTIQASEESAPQGQDSAQDTTSESR.E |
| 3617.5591 | 3616.5518 | 3616.3371 | R.LGSDEDSDDTIQASEESAPQGQDSAQDTTSESR.E + Deamidated (NQ); 2 PhosphoSTY (STY) |

## Table 3: Mass spectrometry analysis of DMP1-C

Few potential phosphorylation sites were identified on the peptide sequences of DMP1-C present in commercial DMP1.

| Observed | Mr(expt) | Mr(calc) | Peptide |
|---|---|---|---|
| 860.3997 | 859.3924 | 859.4399 | K.NIEIESR.K |
| 860.3997 | 859.3924 | 859.4399 | K.NIEIESR.K |
| 873.3964 | 872.3891 | 872.4352 | R.DTGLSQPR.R |
| 873.3964 | 872.3891 | 872.4352 | R.DTGLSQPR.R |
| 1079.4601 | 1078.4528 | 1078.4778 | K.SSSEEDGQLK.N |
| 1920.8580 | 1919.8507 | 1919.9072 | K.SSSEEDGQLKNIEIESR.K |
| 1920.8580 | 1919.8508 | 1919.9072 | K.SSSEEDGQLKNIEIESR.K |
| 2213.0576 | 2212.0503 | 2212.1553 | K.ISILLMFLWGLSCALPVTR.Y + PhosphoSTY (STY) |
| 2367.9465 | 2366.9392 | 2367.0020 | R.ISEEDDRSELDDNNTMEEVK.S |
| 2367.9465 | 2366.9393 | 2367.0020 | R.ISEEDDRSELDDNNTMEEVK.S |
| 2383.9431 | 2382.9358 | 2382.9969 | R.ISEEDDRSELDDNNTMEEVK.S + Oxidation (M) |
| 2657.0991 | 2656.0918 | 2656.1597 | K.ESGENSEQANTQDSGGSQLLEHPSR.K |
| 2657.0991 | 2656.0918 | 2656.1597 | K.ESGENSEQANTQDSGGSQLLEHPSR.K |
| 2680.0994 | 2679.0921 | 2678.9843 | K.LTVDAYHNKPIGDQDDNDCQDGY.- + 4 Deamidated (NQ); PhosphoSTY (STY) |
| 2785.1936 | 2784.1863 | 2784.2546 | K.ESGENSEQANTQDSGGSQLLEHPSRK.I |
| 2785.1936 | 2784.1863 | 2784.2546 | K.ESGENSEQANTQDSGGSQLLEHPSRK.I |
| 2872.2285 | 2871.2212 | 2871.2867 | K.SKESGENSEQANTQDSGGSQLLEHPSR.K |
| 2872.2285 | 2871.2212 | 2871.2867 | K.SKESGENSEQANTQDSGGSQLLEHPSR.K |
| 3617.5337 | 3616.5264 | 3616.3371 | R.LGSDEDSDDTIQASEESAPQGQDSAQDTTSESR.E + Deamidated (NQ); 2 PhosphoSTY (STY) |

### *Responses of odontoblasts in tooth slice cultures to exogenous DMP1and the effect of αvβ3 integrin receptor blockade prior to exposure to exogenous DMP1*

In the organ culture model, expression of hypoxia-inducing factor (HIF)-1α was not detected by real-time qPCR in extracts of microdissected odontoblast layers. This indicated that over the culture period for the studies, the odontoblasts were not exposed to hypoxia (data not shown). In response to exogenous DMP1, odontoblast genes encoding DMP1 and DSPP were down-regulated (Figure 9). The blockade of *αvβ*3 integrin receptors in odontoblasts prior to exposure to exogenous DMP1 enhanced this down-regulation only for the gene encoding DMP1 (Figure 9). Genes encoding TGF-*β*1 and MMP2 were significantly up-regulated in response to the exposure to exogenous DMP1 (Figure 9). There was no significant change in expression of genes encoding TIMP2, MT1-MMP, TLR2 and 4, COL1A1 and COL1A2 in response to the exogenous DMP1(Figure 9) .

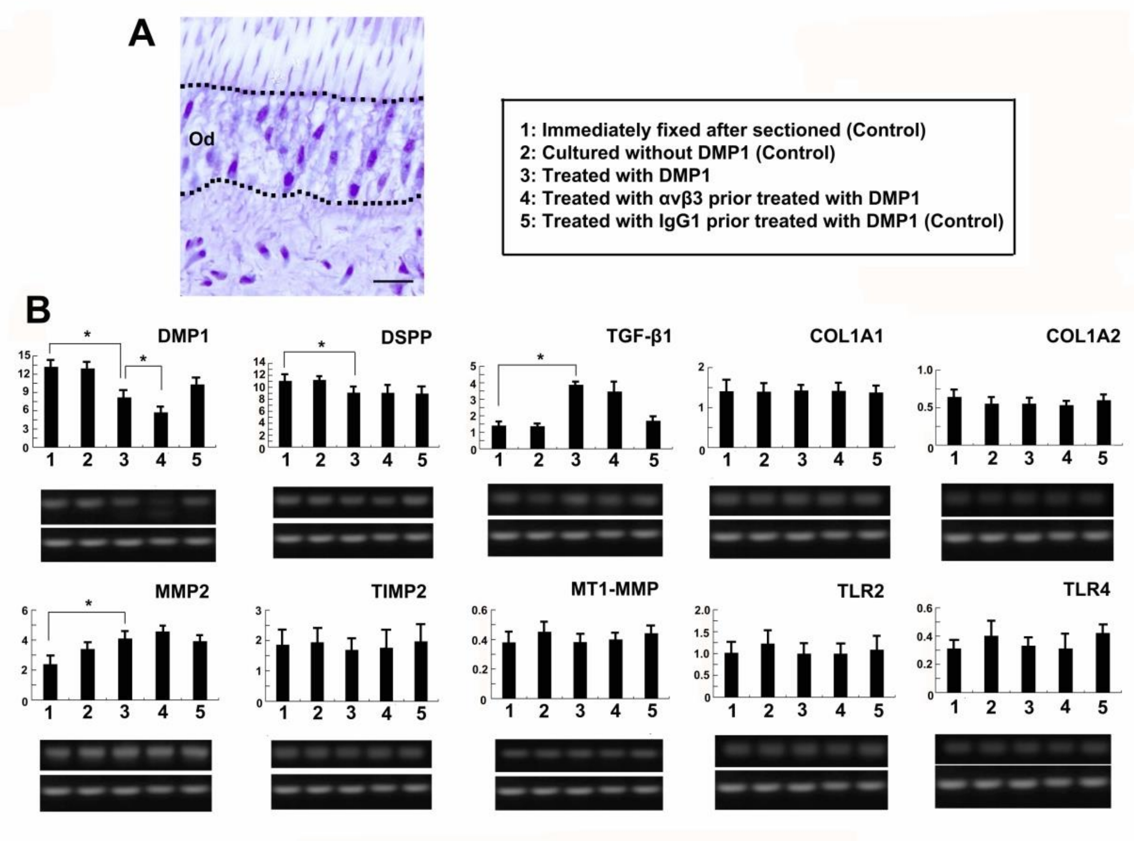


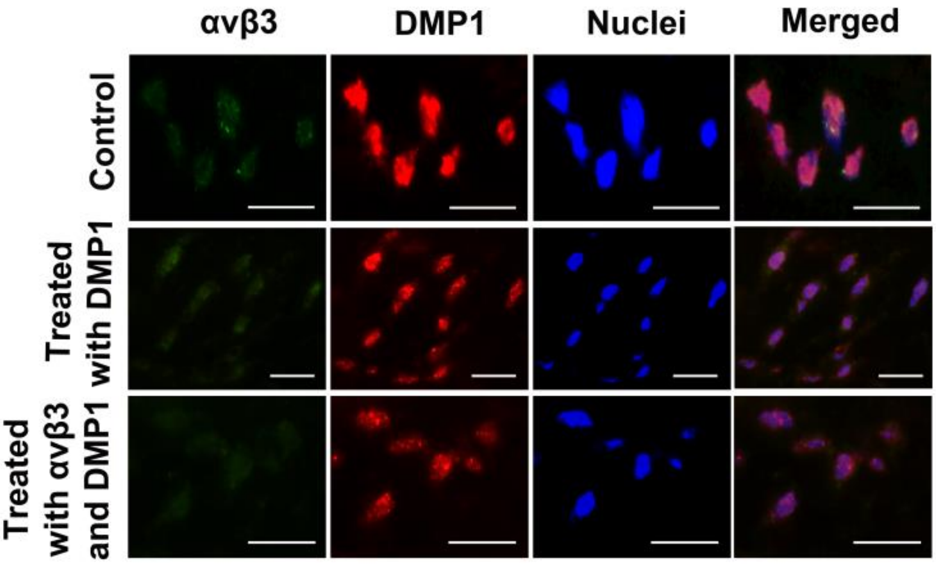
**Figure 9: The synthetic activity of odontoblasts changes in response to exogenous phosphorylated DMP1.** (A) SDS-PAGE and Western blot of DMP1 from commercial recombinant DMP1. Three bands of DMP1, full-length, DMP1-C and –N, were present with weak separated bands above each major band. The immuno-labelled band of DMP1 shows full-length DMP1 and DMP1-C. (B) Toluidine blue staining demonstrates micro-dissection of the odontoblastic layer (Od). Scale bar 30 µm. (C) Bar charts demonstrating significant down-regulation of *DMP1* and *DSPP*. Significant up-regulation of *TGF-β* and *MMP2* was observed. Genes encoding TIMP2, MT1-MMP, COL1A1, COL1A2, TLR2 and TLR4 remain unchanged. The level of mRNA as normalised against *ACTB*. All values depict mean±SD ($n$=3). $^{*}P\leq 0.05$. PCR amplification products of each gene was resolved on 2% agarose gel (first panel under each bar graph) and compared to *ACTB* (second panel under each bar graph).

***Immunofluorescence for DMP1, αvβ3 integrin receptor and GRP78 in odontoblasts from tooth slice cultures***

The intensity of staining for the integrin *αvβ*3 receptor in the odontoblasts from tooth slice cultures exposed to DMP1 was similar to that displayed by healthy teeth (Figure 10). In tooth slices cultured exposed to *αvβ*3 blockade prior to DMP1 addition, there was no signal for integrin *αvβ*3 receptor present (Figure 10).

The d-type distribution pattern of DMP1, that is patterned nuclear staining without cytoplasmic reactivity, predominated in odontoblasts from tooth slice cultures following exposure to DMP1 both with and without blockade of integrin *αvβ*3 (Figure 12).

The intensities of GRP78 from odontoblasts in tooth slice cultures were similar to odontoblasts from healthy teeth.



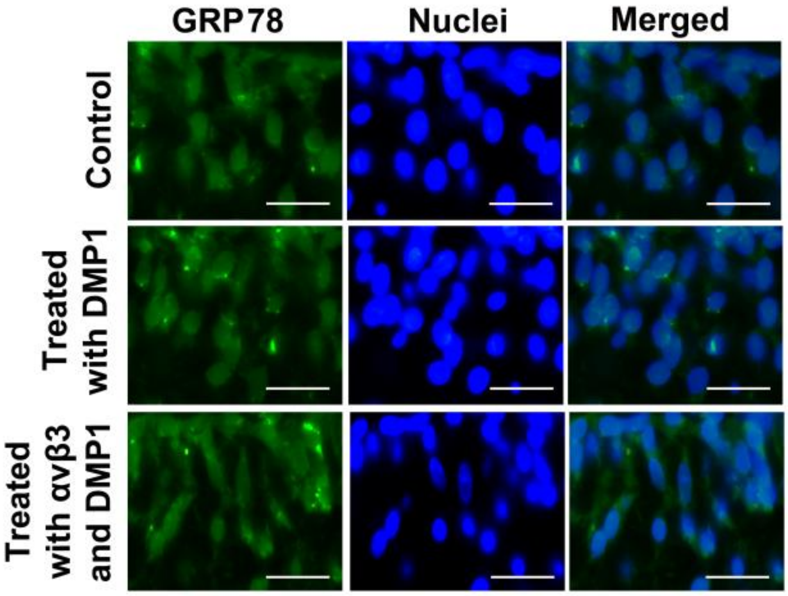


**Figure 10: Immunofluorescence of DMP1, integrin *αvβ*3 and GRP78 in the odontoblast layer following challenge with DMP1.** Odontoblast nuclei were stained with DAPI (third and fourth panels). Sections were dual stained for DMP1 (red fluorescence) and integrin *αvβ*3 (green fluorescence). DMP1 labelling was more intense in odontoblasts from control samples compared to test samples (second panel). Scattered distribution of DMP1 in odontoblast nuclei as observed in test samples (second panel). Integrin *αvβ*3 was very weak in the odontoblast layer from the test sample in which integrin *αvβ*3 receptors were blocked prior to the additional of DMP1 (first panel). Intensities of GRP78 in the test group are similar to GRP78 labelling observed in the control group. Scale bar 30 µm.

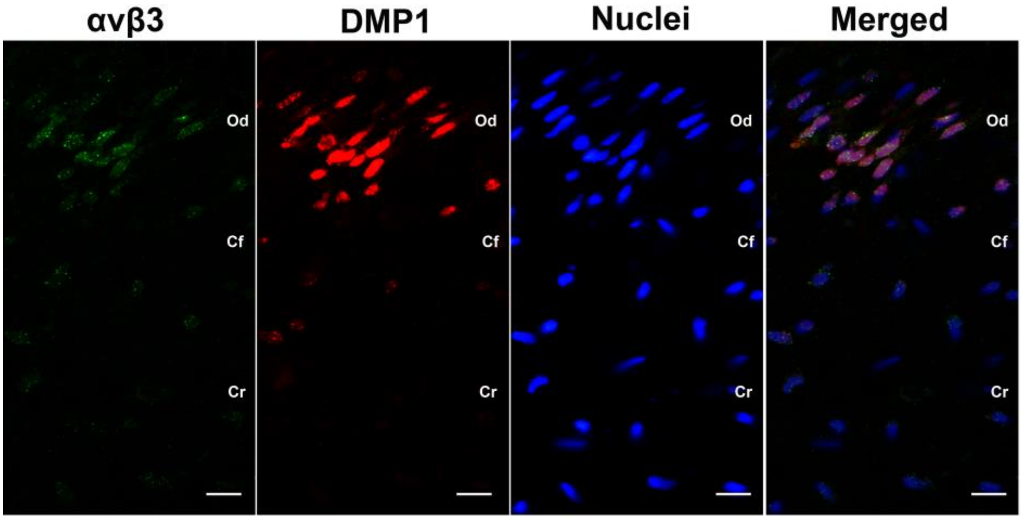


**Figure 11: Immunofluorescence of DMP1 and integrin *αvβ*3 in dental pulp.** Nuclei of cells present in dental pulp including odontoblast layer (Od), cell-free zone (Cf) and cell-rich zone (Cr) were stained with DAPI (third and fourth panel). Sections were dual stained for DMP1 (red fluorescence) and integrin *αvβ*3 (green fluorescence). Immuno-labelling of DMP1 showed specific staining in odontoblasts, particularly in the nuclei. Integrin *αvβ*3 was more intense in the odontoblast layer (Od) with only trace scattered distribution observed in the cell-rich zone (Cr). Scale bar 30 µm.

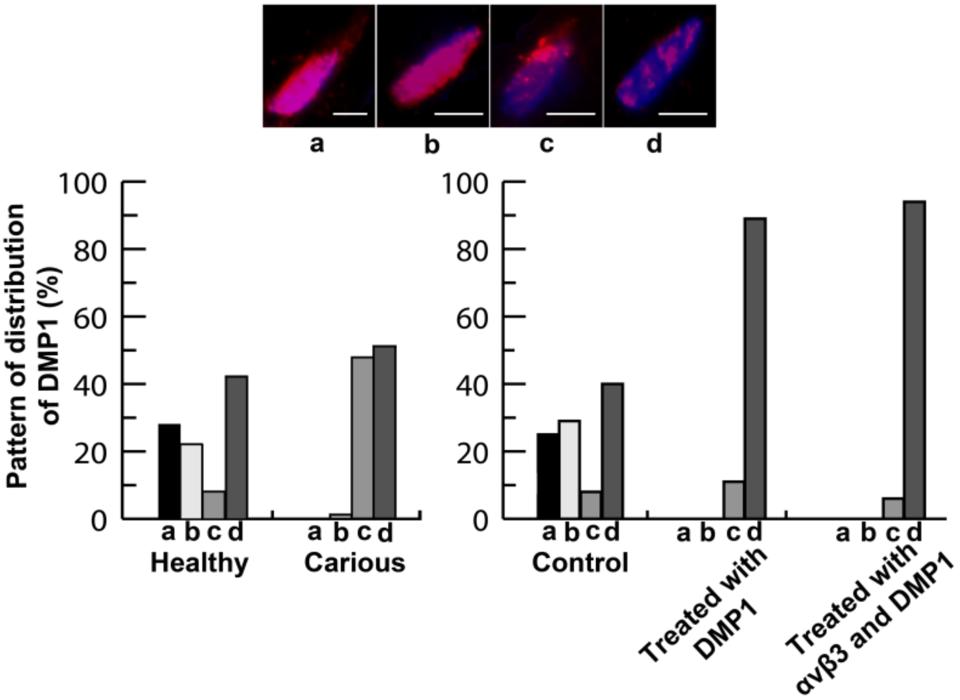


**Figure 12: Bar charts representing percentage of different distribution patterns of DMP1 in odontoblasts.** The distribution of DMP1 in odontoblasts was divided into 4 groups according to different patterns of distribution in cytoplasm and nuclei of odontoblasts as follows;

**a** = uniformly distributed inside the nucleus with presence in the cytoplasm, **b** = uniformly distributed inside the nucleus without presence in the cytoplasm, **c** = non-uniformly distributed inside the nucleus with presence in the cytoplasm and **d** = non-uniformly distributed inside the nucleus without presence in the cytoplasm. In healthy teeth, the distributions pattern was similar to that of the control sample from the culture experiment. The **a** and **b** types were markedly down-regulated in carious teeth with the increase of **c** and **b** types. The same distribution pattern was observed in culture samples exposed to DMP1. Scale bar 10 µm.

# Discussion

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The aim of this study was to explore the possible role of exogenous DMP1 released from demineralised carious dentin in the adaptive response of human odontoblasts to microbial invasion of dentin. Immunofluorescences staining for DMP1 on human odontoblasts obtained from four different stages of carious teeth revealed the response of odontoblasts start since carious process reach dentin front. This confirmed by the presence of reactionary dentin in early dentin carious teeth but not enamel carious teeth according to the connection of dentinal tubules and odontoblastic processes along dental pulp to dentinoenamel junction (DEJ). Late stage of carious teeth which caries extend more than 2/3 of dentin showed the necrosis of dental pulp caused by infection through breakdown dentin. Therefore, this study focuses on the adaptive response of odontoblasts from carious teeth which carious process occurs from 1/3 to 2/3 of dentin.

DMP1 plays an essential role in biomineralisation by functioning both as a nucleation site for mineral deposition but also as a transcription factor regulating genes involved in biomineralisation [5, 10]. The proteolytically cleaved products of DMP1, DMP1-N and -C, are likely to perform distinct functions according to their different distribution in tissues and cell compartments [15, 39]. Only trace amounts of DMP1-C were detected in Rd by Western blot of extracted matrix while immuno-gold labelling failed to detect DMP1 in Rd. These findings support a marked depletion of DMP1 in Rd [40]. The gene encoding DMP1 was also reported to be markedly down-regulated in the odontoblast layer adjacent to carious lesions during Rd formation [35]. The lack of DMP1 potentially explains the irregular dentinal structure with fewer tubules and tubular constrictions observed in Rd [33]. Of note, *DMP1*-deficient mice were reported to show altered dentinal structure with fewer dentinal tubular branches [17]. This adaptive response of odontoblasts is potentially affected by exogenous regulators released during carious progression. These include bacterial products and mobilised exogenous dentin proteins released from demineralised dentinal matrix [34, 41, 42]. DMP1 was observed to be localised in peri-tubular dentin and could be mobilised when demineralisation of dentin by acidic bacterial products occurs. This is supported by Western blot of DMP1 indicating relatively large amounts of DMP1-C present in extracted dentin proteins from carious lesions compared to sound dentin from healthy teeth. The full-length rat DMP1 has 65 Ser/Thr potential phosphorylation sites [5] from 22 sites in $NH_2$-terminal and the COOH-terminal having 43 [12, 43]. DMP1 from mammalian tissue is predicted to be heavily phosphorylated [44, 45]. However, Western blot for phosphorylated serine suggests partially phosphorylated DMP1 in carious dentin. The de-phosphorylatation of DMP1 in carious dentin is credibly explained by the increased phosphatase activity during carious progression [46, 47]. Therefore, the phosphorylated DMP1 embedded in surrounding hydroxyapatites could, when immobilised following demineralisation due to the carious process, be de-phosphorylated by active phosphatases. During carious progression, odontoblast processes remain inside the tubules and are believed to be the conduits for external stimuli. The potentially significant amount of phosphorylated DMP1-C released from dentin due to the carious process could influence the adaptive response of odontoblasts as DMP1-C is distributed in the nuclei of cells involved in active biomineralisation, suggesting a significant biological function [39].

Comparison of the immunofluorescence for DMP1 between odontoblasts from healthy and carious teeth indicates a marked change of distribution. DMP1 was more intense inside the nucleus with scattered distribution in cytoplasm of odontoblasts from healthy teeth. The distribution pattern of DMP1 in the nuclei of odontoblasts from carious teeth was less uniform than that observed for healthy teeth. Transcriptional regulators function by the ability to reach the nucleus or to remain in the nuclear compartment [48-52]. Additionally, non-uniform nuclear distribution, for instance in nuclear speckles, of some transcription factors, reflects targeting to specific sites of active transcription [53]. Therefore, the presence of patterned distribution of DMP1 in the nuclei of odontoblasts from carious teeth suggests a potential role of DMP1 as a transcription factor during the formation of reactionary dentin. Interestingly, challenge with exogenous, partially phosphorylated DMP1 in the tooth slice culture model caused significant change in the distribution pattern of DMP1. It was noteworthy that the profile of staining shifted abruptly to correspond closely with that observed for odontoblasts adjacent to microbial invasion of dentin. This provided evidence for both the plausibility of the hypothesis and for the suitability of the experimental model to study functionality of polarised odontoblasts. The gene encoding DMP1 was significantly down-regulated in response to exogenous DMP1 in tooth slice cultures. This phenomenon is similar to the marked down-regulation of DMP1 at both the gene and protein levels during carious progression and reactionary dentin formation. However, the gene expression level of DMP1 from the odontoblast layer in tooth slice cultures exposed to exogenous DMP1 was not as low as previously detected in carious teeth. This could be due to shorter duration of exposure to exogenous DMP1 in tooth slice culture than in the chronic carious process. DMP1 mediates calcium release [54] and promotes nucleation and growth of hydroxyapatite crystals in the extracellular matrix [55, 56]. Therefore, accumulation of DMP1 inside the nucleus during carious progression and reactionary dentin formation with reduced secretion into the extracellular matrix, potentially limits calcium deposition in reactionary dentin. This is supported by the reduced hardness reported for this matrix [57]. Expression of genes encoding DSPP, TGF-$\beta$1 and MMP2 in odontoblasts was also significantly altered by exposure to exogenous DMP1 in tooth slice cultures. The gene encoding MMP2 was significantly up-regulated in tooth slice cultures exposed to DMP1, similar to the reported up-regulation of MMP2 during reactionary dentin formation [33]. However, expression of *TIMP2* and *MT1-MMP* remained unchanged in tooth slice cultures whereas products of these genes were reported to be up-regulated during reactionary dentin formation in response to polymicrobial invasion [33]. This suggests the specific adaptive response of odontoblasts to DMP1. During polymicrobial invasion of dentin, several factors are involved in the regulation of odontoblast responses including bacterial products and mobilised proteins released from demineralised dentinal matrix [34, 41, 42]. These complexities are credibly competing signals that regulate the specific responses of odontoblasts. This potentially explains the different expression pattern of *DSPP* and *TGF-β1* observed in the tooth slice cultures exposed to DMP1 from the reported gene expression pattern during carious progression and reactionary dentin formation [33, 35]. In addition, the genes encoding TLR2, 4, COL1A1 and COL1A2 remained unchanged in tooth slice cultures exposed to DMP1.

Integrin *αvβ*3, a receptor reported to bind DMP1 and mediate signal transduction through cell surface interactions [24], shows increased expression on the surface of odontoblasts from carious compared to healthy teeth. This receptor was first characterised as the vitronectin receptor, but presents a wide spectrum of binding for various extracellular ligands [58]. The activation of integrin *αvβ*3 regulates cell homeostasis [24] and contributes to the interaction with the cytoskeleton in the continuous re-organisation of actin microfilaments thus maintaining and stabilising the cells [59]. Accordingly, increased expression of *αvβ*3 integrin in the odontoblast layer from carious teeth indicates the required maintenance and stabilising of odontoblasts during reactionary dentin formation [59]. Following interaction with a DMP1 preparation similar to that used in the present study [22], integrin *αvβ*3 mediates transcription factor activity in cells committed to active biomineralisation [24]. From the present study, exogenous partially phosphorylated DMP1 mediates changes in expression of *DSPP*, *TGF-β1 and MMP2* via an *αvβ*3 integrin-independent pathway as blockade of the *αvβ*3 integrin prior to addition of phosphorylated DMP1 did not affect the response pattern. Interestingly, expression of *DMP1* was further down-regulated in the group that was blocked with anti-*αvβ*3 integrin prior to addition of phosphorylated DMP1. This suggests other potential competing receptors on the odontoblast surface that are capable of binding DMP1 and mediating the signal to down-regulate *DMP1.*

Glucose-regulated protein 78 (GRP78), one of the endoplasmic reticulum (ER) chaperone proteins, was reported to mediate endocytosis of DMP1 in odontoblasts and osteoblasts [60]. GRP78 is considered to be a central regulator of ER homeostasis according to its multifunctional roles in protein folding, and ER calcium binding; also regulation of the activation of transmembrane ER stress sensors [61]. Accordingly, GRP78 is believed to play a critical role in the survival of the cells during stress conditions by suppressing stress-induced apoptosis [62-64]. GRP78 is also reported to occur extracellulary and to be involved in the nucleation of calcium phosphate on collagen fibrils [38]. The immunofluorescence of GRP78 was increased in odontoblasts from carious teeth during reactionary dentin formation compared to healthy teeth. This demonstrates the potential role of GRP78 in the regulation of odontoblast stress during rapid transportation of calcium to the extracellular matrix during formation of reactionary dentin in carious teeth [38, 65]. We have reported previously increased collagen type I synthesis and the uniform collagen framework for reactionary dentin formation [33]. GRP78 is also one the chaperones involved in assisting the correct folding of collagen [66, 67]. Therefore, increased GRP78 during reactionary dentin formation is potentially involved in the formation of the collagen scaffold. Up-regulation of GRP78 results in intracellular calcium fluxes [38, 60]. The severe down-regulation of DMP1 in tooth slice cultures in which $\alpha v\beta 3$ integrin was blocked prior to exposure to DMP1 supports indirectly, the potential intimate role of GRP78 in odontoblasts in mediating DMP1 action. While GRP78 expression showed no direct relationship to the alteration of *DSPP*, *TGF-β1* and *MMP2* expression, the failure of $\alpha v\beta 3$ blockade to modify gene response patterns supports the specific role of GRP78 in regulating odontoblast responses to exogenous, partially phosphorylated DMP1.

Signal transduction pathways modified by the altered extracellular environment target transcription factors, transcriptional co-regulators, and chromatin-modifying factors, leading to phosphorylation or de-phosphorylation events [68-70]. These modifications cause either positive or negative regulation of the activity of transcription factors, thereby producing changes in the secretory behaviour of the cells [68]. Transcription factors shuttle between cytoplasmic and nuclear compartments depending on phosphorylation status [71]. This cascade was observed in carious teeth and tooth slice cultures challenged with DMP1. A patterned nuclear distribution of DMP1-C was observed in the cultured cells [39]. A consistent finding in the present study was that DMP1 was not observed in cytoplasm but formed a pattern in the nuclei of reactive odontoblasts. This suggests the activity of the putative transcription factor has been modified. To date, the binding capacity of GRP78 to BMP1-cleaved C-terminal fragment of DMP1 both phosphorylated and non-phosphorylated forms along with the signal transduction of GRP78 in response to exogenous DMP1, are not clearly understood. Both DMP1 extracted from the dentin layers and commercial DMP1, were predominantly DMP1-C, which contains nuclear localisation signals (NLSs) [60]. DMP1-C was found accumulated in the nuclei of cells committed to biomineralisation while DMP1-N was not detected [39]. Interestingly, DMP1-C shows a lack of classical DNA-binding domains which implies a complex nuclear function [39]. Accordingly, the change of odontoblast behaviour in response to mobilised DMP1-C from carious dentin and DMP1-C contained in the commercially available product, credibly reflect an indirect regulation [38, 60]. The internalisation of phosphorylated or partially phosphorylated DMP1-C derived from carious dentin and commercial DMP1 in odontoblasts has not been confirmed. Further study of this mechanism, by addition of fully phosphorylated/ non-phosphorylated forms of fluorochrome-labeled of BMP1-cleaved C-terminal of DMP1 to tooth slice cultures, should be considered to explore this regulatory pathway.

# Conclusion

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This study demonstrates an effective organ culture model for study of the response of human odontoblasts to the critical exogenous stimuli released in the carious process as displayed in Figure 10. The data provided highlight the critical role of exogenous DMP1 during the carious process and reactionary dentin formation. The knowledge obtained from this study provides a basis for the future treatment protocols using DMP1 in vital pulp therapy.

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# Output

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## International journal publication

The data is now dividing into two parts and planned to submit to the following journals within August, 2015;

1. BONE (5-year Impact factor 4.312)

2. Caries research (Impact factor 2.73)

## Presentation

1. Oral presentation in “Sydney university - Tohoku university, research collaboration” at The University of Sydney, Australia on the topic “Reactionary dentin formation and its structure” in 2014
2. Oral presentation to dentists on continuing professional development course (Sukothai hospital, Thailand) on the topic “Advance in Operative Dentistry” in 2014
3. Oral presentation in “Continuing education on mineralized tissue research” at Faculty of Dentistry, University of Geneva, Switzerland in 2014