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Research Article

# Expression of Calcium Sensing Receptors Increases in Differentiated Human Odontoblasts at Stable Extracellular Calcium

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## Abstract

The calcium-sensing receptor (CaSR) is a G protein-coupled receptor responsible for calcium (Ca2+) homeostasis and translating changes of extracellular Ca2+ into intracellular signalling pathways. Odontoblasts are specialised cells in the dental pulp that produce and regenerate dentin. Increased expression of CaSR in odontoblasts, which was up regulated by elevated extracellular Ca2+, was reported, however, whether expression of CaSR changes during differentiation of dental pulp cells when extracellular Ca2+ concentration is constant remains unclear. We have used immunocytochemistry (ICC) to measure levels of CaSR expression and electrophysiology to evaluate the cell capacitance in human telomerase reverse transcriptase dental pulp (hTERT DP) cells that were non-differentiated and differentiated in vitro into odontoblasts. hTERT DP-derived odontoblasts demonstrated prominent changes in cell morphology upon differentiation, and occupied lager area without changes in the cell capacitance. Semi quantitative analysis showed that normalized fluorescence intensity of CaSR immunostaining was doubled in differentiated hTERT DP-derived odontoblasts when compared to non-differentiated hTERT DP cells. In addition, increased CaSR expression was evident in cellular processes and on the edges of differentiated odontoblasts. To conclude, the expression levels and cellular localization of the CaSR changes during in vitro odontoblast differentiation in stable extracellular Ca2+, which suggests its pivotal role during dentinogenesis.

**Abbreviations:** CaSR: Calcium-Sensing Receptor; DP: Dental Pulp; PFA: Paraformaldehyde; PBS: Phosphate Buffered Saline; ICC: Immuno Cyto Chemistry; BS: Bovine Serum Albumin

## Introduction

Extracellular calcium ions ($Ca^{2+}$) promote osteogenic differentiation and mineralization of human dental pulp cells [1,2]. The calcium-sensing receptor (CaSR) is a G protein-coupled receptor mainly responsible for the maintenance of $Ca^{2+}$ homeostasis and tissue mineralization [3,4]. It was recently shown that CaSR signaling promotes differentiation of dental pulp (DP) cells into odontoblasts [5]. Odontoblasts are highly specialized polarized cells, arranged as a single layer of palisade cells between the dental pulp (DP) and dentin in the tooth [6]. Their cell bodies cover the surface of the DP and long cellular processes are projected into the dentin [7]. The DP contains stem cells that possess the ability to differentiate into odonto blast-like cells [1]. Dentin formation (dentinogenesis) is a mechanism performed by the odontoblasts, which encompasses construction of the dental tissue [1]. The function of odontoblasts is not limited to the primary dentinogenesis, but also includes maintenance and regeneration of dentin during pathological conditions to prevent DP exposure [7]. The CaSR expression is dynamic during development and disease and is tightly controlled by multiple signalling pathways [8]. The aim of this study was to investigate how expression of CaSR changes in developing odontoblasts when extracellular $Ca^{2+}$ is constant and close to the foetal (1.8mM).

## Materials and Methods

Human telomerase reverse transcriptase dental pulp immortalised cells (hTERT DP) previously developed by Kitagawa [9] were used as a cell model for this study. Minimum Essential Medium Eagle Alpha Modification (α-MEM) (Thermo Fisher Scientific), that

contains 1.8mM Ca2+, supplemented with 10% foetal bovine serum (FBS, Sigma Aldrich) and 1% penicillin/streptomycin (5000U/mL; GibcoTM) was used to grow and maintain the cells. hTERT DP cells were differentiated using mineralization-inducing media that is based on α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 50μg/ml ascorbic acid (Sigma Aldrich), 10mM/L β-glycerophosphate (Cayman) and 0.1μM/mL dexamethasone (Sigma Aldrich).hTERT DP-derived odontoblasts were examined before and after 7 days of treatment with mineralisation-induced media. Fixation was performed to preserve cell morphology of hTERT DP cells using 4% paraformaldehyde (PFA) (Sigma-Aldrich) [10].

For immunocytochemistry (ICC), fixed cells were incubated in blocking/permeabilisation solution composed of phosphate buffered saline (PBS) (Sigma-Aldrich) + 1% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% Tween20 (Sigma-Aldrich). Antibodies were diluted in blocking/permeabilisation solution to prevent antibody aggregation. Primary antibodies were mouse CaSR (Thermo Fisher Scientific) and rabbit α-tubulin (GeneTex) was employed as a reference generic structural protein. Secondary antibodies were donkey anti-rabbit Alexa Fluor 594 and donkey anti-mouse IgG Alexa Fluor 488, (Thermo Fisher Scientific both). Vectashield hard set mounting medium with DAPI (Vector Laboratories) was applied to each coverslip, followed by addition of 50 μ L drop of PBS-glycerol. Negative controls were prepared by incubating the cover slips with hTERT DP in blocking buffer without the primary antibodies, followed by staining with secondary antibodies. The slides with stained hTERT DP cells were analysis using fluorescent microscope Olympus BX61 with x20 objective and Olympus XM10 monochrome camera.

Cell capacitance of non-differentiated hTERT DP cells and hTERT DP-derived odontoblasts was recorded using the patch-clamp voltage-clamp conventional whole-cell technique. The bath solution contained (in mM): 144 NaCl (Thermo Fisher Scientific), 2.5KCl (Thermo Fisher Scientific), 0.5MgCl2 (Sigma-Alrich), 2 CaCl2 (Sigma-Aldrich), 10 D-glucose (Thermo Fisher Scientific), 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Thermo Fisher Scientific); pH was adjusted to 7.4 using 1M NaOH (Thermo Fisher Scientific). The pipette solution contained (in mM): 140 KCl, 6 NaCl, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3 Na2-ATP (Sigma-Aldrich), 1 Na-GTP (Sigma-Aldrich), 3MgCl2, 1 CaCl2 and 5 ethylene-glycol-tetra-acetic acid (EGTA, Thermo Fisher Scientific); pH was adjusted to 7.2 using 1M KOH (Thermo Fisher Scientific). Pipette resistances were ~3-5 MΩ when filled with the pipette solution. Cell capacitance measurements were performed using Axopatch 200A amplifier and Digidata 1440 A/D interface (Axon instruments, Forster City, CA, USA) and pipette holder optimized for low noise recordings (G23 instruments, UCL) at a controlled room temperature of 22±0.5°C. Cell capacitance measurement was performed using Clampex 10.2.

Semi quantitative analysis of the images was performed using ImageJ software (NIH) which allowed the quantification of fluorescence intensity of CaSR and α-tubulin staining in the cells. The data were quantified using Microsoft Office Excel 2013 and Microcal Origin 6.0 software. The patch-clamp data were analysed using Clampfit 10.2. Statistical analysis was performed using unpaired t-test; the means were considered as significantly different if $P < 0.05$. N – number of images, used for statistics and calculation of significance; n – number of cells.

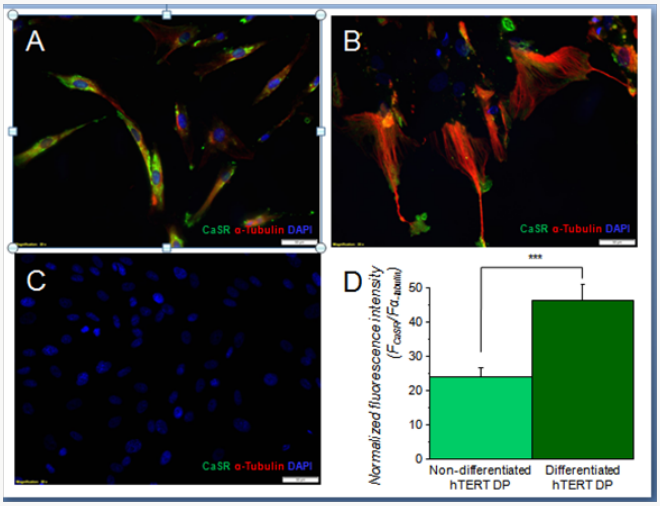


**Figure 1:** Expression of CaSR in huExpression of CaSR in human telomerase reverse transcriptase dental pulp (hTERT DP) cells before and after differentiation into odontoblasts. dontoblasts.
Immunocytochemistry showing expression of CaSR (green) and a-tubulin (red) in non-differentiated hTERT DP cells (A), differentiated hTERT DP cells into odontoblasts (B), and negative control non-differentiated in hTERT DP cells (C); negative control for hTERT DP-derived odontoblasts was similar to C (data not illustrated). DAPI was used to stain nucleus of the cells. Bar chart graph showing mean values ± standard error of mean of normalized fluorescence intensity ($F_{CaSR}/F\alpha\text{-}_{tubulin}$; D). P values represent unpaired t-test, where *** - $P < 0.001$.

## Results

Differentiation of hTERT DP cells into odontoblasts resulted in several morphological transformations, i.e. changes of the cell shape, localization of the nucleus, localization and levels of the CaSR staining. Non-differentiated hTERT DP cells have spindle shape with central nucleus and CaSR expression dispersed across the whole cell (Figure 1A). Most of the hTERT DP-derived odontoblasts were flat irregular-shaped with elongated single cellular processes with nuclei predominantly located at the periphery; the CaSR staining was present mainly at the cellular processes and on the edges of the cells (Figure 1B). Interestingly, the nuclei of hTERT DP-derived odontoblasts were located predominantly at the opposite side to the cellular processes. The CaSR and α-tubulin fluorescence intensity was calculated by subtracting the negative control fluorescence (Figure 1C). The mean area of a single hTERT DP-derived odontoblast was by 1.6 bigger in comparison to the mean area of a single non-differentiated hTERT DP cell ($P<0.0001$).

At the same time, the absolute intensity of α-tubulin fluorescence in non-differentiated hTERT DP cells was by 1.6 greater than that of hTERT DP-derived odontoblasts ($P<0.0001$). Besides that, cell capacitance of non-differentiated hTERT DP cells and differentiated hTERT DP-derived odontoblasts were similar: 56.6±12.0 pF (n=6) and 56.6±10.0 pF (n=7), respectively, which suggests that mineralisation-induced media did not change the size of hTERT DP cells. Assuming that in the cells with similar membrane square surface and, respectively, volume characteristics expression of structural α-tubulin protein is constant the fluorescence intensity for CaSR was normalised to α-tubulin. Normalised fluorescence intensity (FCaSR/Fα-tubulin) was 23.8±2.7 (N=18; n=296) and 46.1±4.7 (N=17; n=248) for non-differentiated hTERT DP cells and hTERT DP-derived odontoblasts, respectively, showing a significant two-fold increase of CaSR expression ($P<0.001$) stimulated by mineralization-inducing media (Figure 1D).

## Discussion

This study shows that odontoblasts differentiation causes multiple transformations in hTERT DP cells. The development of cellular processes and cell polarization are one of the main morphological features of the differentiation [11]. It was shown earlier that following differentiation, the hTERT DP-derived odontoblasts become polarised: their nuclei are aligned away from the newly formed dentin and relocated to the periphery [12,13]. Differentiated odontoblasts also possess single processes that extend deeply into the dentin. Our findings are concordant with those ones as the hTERT DP-derived odontoblasts show pronounced single cellular process and peripheral localization of nuclei. Also, differentiation promotes the development of flat irregular-shaped cells from spindle shaped cells, which could be explained by the current understanding that secretory odontoblasts exhibit an elongated columnar shape and are densely packed next to each other in DP [14].

Differentiated hTERT DP odontoblasts show increased expression of CaSR at the cellular process, suggesting that the CaSR is involved in dentin production as the processes project into the dentin tubular structure [7]. At the same time, dispersed CaSR expression in paranuclear area of non-differentiated hTERT DP cells is in accordance with a study showing that CaSR is constitutively expressed in the cytoplasm of human dental pulp cells [5]. Odontoblasts actively transport $Ca^{2+}$ towards the site of mineralisation, resulting in an extracellular $Ca^{2+}$ accumulation [15]. Recent studies show that treatment of human dental pulp cells with 2mM $CaCl_2$ increased immuno positive reactions with anti-CaSR antibody, suggesting that CaSR expression is up regulated by $Ca^{2+}$ in order to promote dentin formation [5]. At the same time, our studies suggest that increase of extracellular $Ca^{2+}$ that is independent on other factors is not sufficient to facilitate CaSR expression in human DP cells.

Analysis of the average single cell surface together with assessment of absolute fluorescence intensity of α-tubulin and electrophysiological analysis of cell capacitance suggest that treatment of hTERT DP cells with mineralisation-inducing media influences only dimensional characteristics of hTERT DP cells whereas the size of the cells remains constant. Since fluorescent analysis of the protein expression is a mean function to the cell surface and due to the proportional expression of α-tubulin did not change during odontoblast differentiation, we performed our semi-quantitative analysis for CaSR as normalised fluorescence intensity to that of α-tubulin ($F_{CaSR}/Fα\text{-}_{tubulin}$). The resultant relative expression of CaSR was two-fold significantly higher in hTERT DP-derived odontoblasts vs. non-differentiated hTERT DP cells. This study demonstrates that CaSR expression is increased and allocated to the cellular processes and edges following odontoblast differentiation when extracellular $Ca^{2+}$ is stable and close to the foetal. The cellular processes of odontoblasts project into the dentin, therefore, the CaSR may be involved in a cascade of dentin formation and regeneration. Further investigation using functional readouts and specific CaSR pharmacology is in progress to prove this concept.

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