Role of HERPUD1 protein as modulator of vascular calcification

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INTRODUCTION

Calcifications correspond to accumulation of calcium-phosphate crystals in different tissues. Normally occurs during bone mineralization, or abnormally in soft tissue as vessels, where vascular smooth muscle cells (VSMC), among other mechanisms, acquire an osteoblast-like phenotype promoting the extracellular matrix secretion and pathological accumulation of calcium crystals on tunica media.

The function of every specialized secretory cell involves a high demand of endoplasmic reticulum (ER) function and therefore requires strict control of proteostasis. The ER membrane protein HERPUD1 is a key component of the ER-associated degradation and proteostasis control. We have demonstrated its importance in mineralization processes of bone cells in vitro. The aim of this study was to establish whether HERPUD1, as part of secretory pathway, play a role in VSMC calcification in vitro.

METHODS

Cell culture. Primary rat VSMC and cell line A7R5 were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 50 µg/mL penicillin/streptomycin.

Determination of calcification. VSMC and A7R5 cells were undergo mineralization conditions, high Pi-Ca^{2+} medium (Na_2HPO_4/NaH_2PO_4 3mM and 2,7 mM $CaCl_2$), in presence and absence of HERPUD1 for 7 days. Calcium deposits levels were evaluated through alizarin-red staining.

Collagen and non-collagenous proteins (NCP) secretion were determined through Sirius Red/Fast Green Collagen Staining Kit (Chondrex).

Determination of mRNA. Expresion of HERPUD1 and osteo-conversion gene levels was determined by qPCR. Using M-MLV Reverse Transcriptase (Promega) for the synthesis of cDNA and Brilliant III ultra-fast SYBR master mix (Agilent) for amplification. The relative expression of each mRNA was determined using the ΔΔCt method.

Determination of protein expression. Expression of HERPUD1 were determined by western blot. A total of 30 µg of protein was electrophertically resolved in a 10% Acrylamide/-Bis-acrylamide gel and transfered to a PVDF membrane. After blocking, membranes were incubated with primary antibodies O.N. Secondary HRP-linked antibody was used by 1 hr. After washing Westar Supernova was used as chemiluminiscent substrate. Images were obtained in a C-DiGit® Blot Scanner (Li-Cor).

Statistic analysis. The results are shown as a mean ± S.E.M. n: 3-5 independent experiments (unless otherwise is indicated), $p<0.05$ was set as significative. One-way ANOVA analyses was used according to the experimental settings, with Dunnette's post-hoc test. Two-way ANOVA analyses was used according to the experimental settings, with Tukey post-hoc test.

CONCLUSIONS

Our results show that HERPUD1 is important for mineralization processes and may be useful new target to understand, diagnostic or treatment of mineralization disorders.

FUNDINGS

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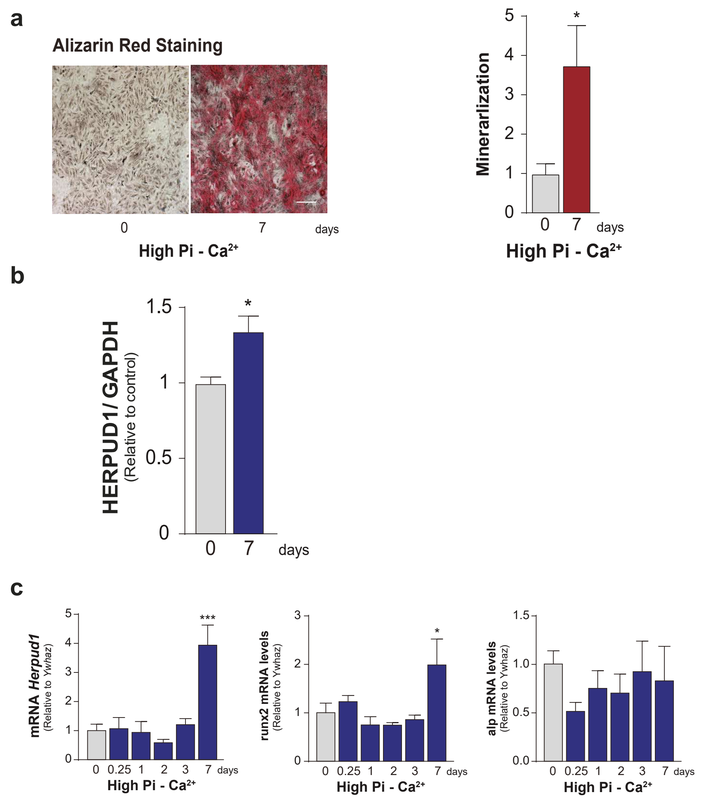
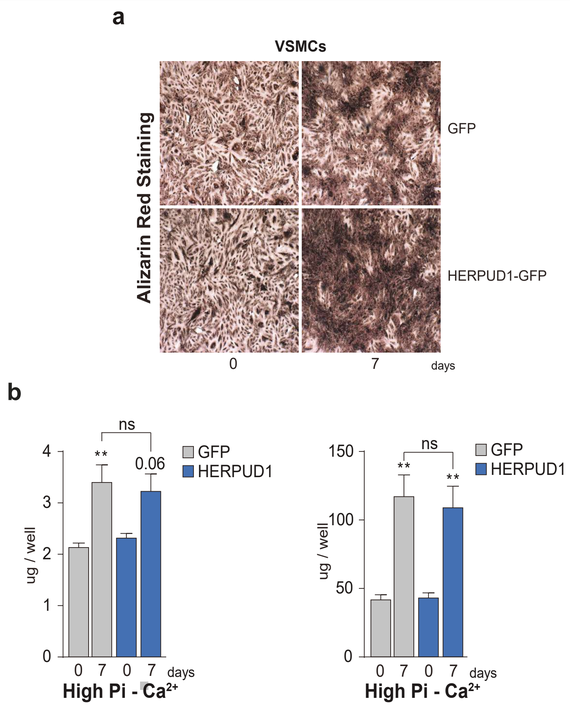
RESULTS

FIG 1. CALCIFICATION A7R5 IS ACCOMPAINED BY INCRESE IN HERPUD1 EXPRESION



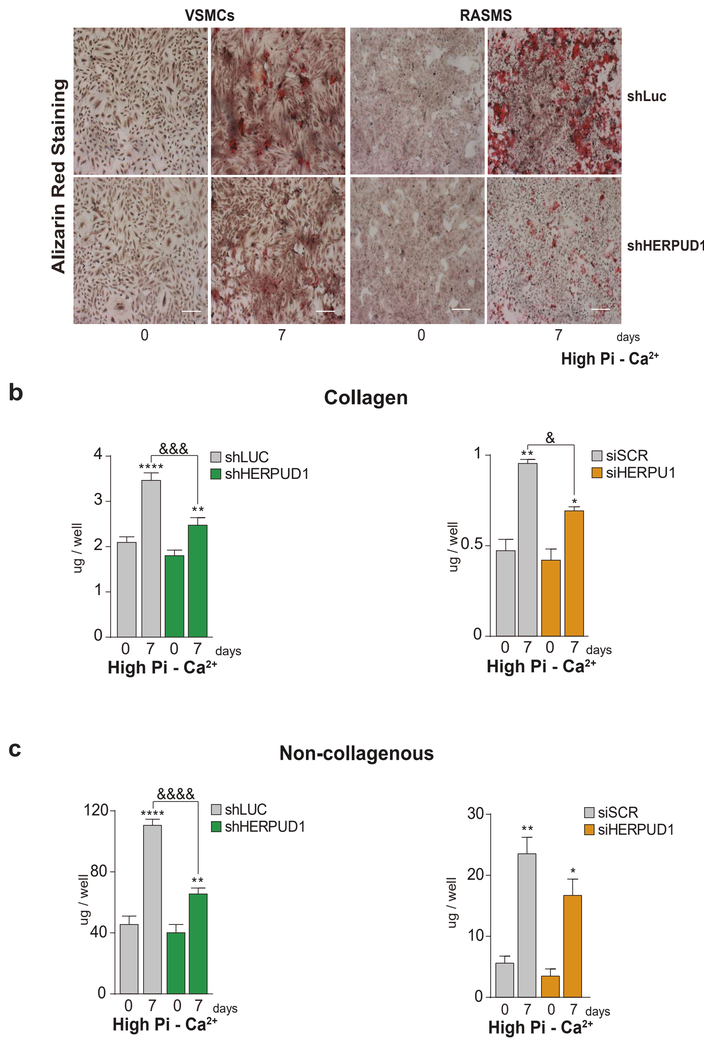
a. A7R5 cells were treated for 7 days with high Pi-Ca^{2+} (Na_2HPO_4/NaH_2PO_4 3mM and 2,7 mM CaCl2) media. Mineralization was visualized with Alizarin Red staining and calcium deposits stained were dissolved and quantified. **b.** HERPUD1 protein were determined by Western blot in A7R5 cells subjected to 7 days to high Pi-Ca^{2+} **c.** HERPUD1, Runx2 al ALP mRNA levels were determined by RT-qPCR in A7R5 cells subjected to 6 h, 1, 2, 3 and 7 days underwent high Pi-Ca^{2+}.

FIG 3. EFFECT OF HERPUD1 OVEREXPRESSION



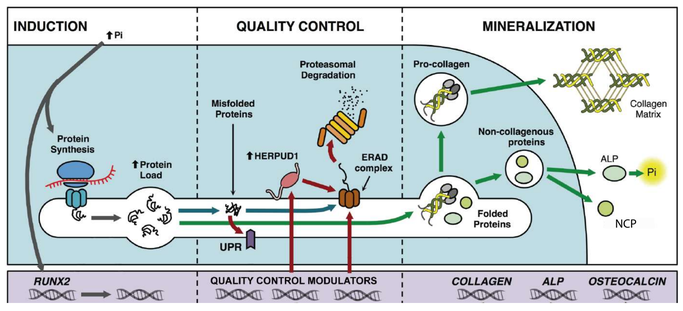
a. A7R5 cells were transduced with HERPUD1-GFP and GFP constructs and 72 h post-transduction were treated for 7 days with high Pi-Ca^{2+} media. Mineralization was visualized through calcium deposits stained with Alizarin Red. Images are representative of n=3. **b.** A7R5 cells were transduced with HERPUD1-GFP and GFP constructs. Cells were subjected to high Pi-Ca^{2+} media for 7 days. Collagen secretion and non-collagenous proteins secretion were evaluated using the Semi-Quantitative Tissue Total Collagen Staining Kit. Mean ± SEM of n=5. Statistical significance was calculated using two-way ANOVA. **p<0.01 vs. same genetic background non-treated cells.

FIG 2. EFFECT OF HERPUD1 REDUCTION ON MINERALIZATION OF VASCULAR CELLS



a. Rat primary VSMC culture (RASMS) and A7R5 cells were transduced with lentivirus overexpressing a shRNA against HERPUD1 and a shRNA control against Luciferase. 48 h post-transduction, cells were selected with 5 ug/ml puromycin. shHERPUD1 and shLUC cells were subjected to mineralization conditions for 7 days and stained with Alizarin Red. Images are representative of n=3. **b.** A7R5 cells were transduced with lentivirus overexpressing a shRNA against HERPUD1 and a shRNA control (shLUC), and siRNA Scramble (siSCR) and siRNA against HERPUD1 (siHERPUD1). In case of lentivirus, 48 h post-transduction, cells were selected with 5 ug/ml puromycin. Cells were subjected to high Pi-Ca2+ media (Na2HPO4/NaH2PO4 3mM and 2,7 mM CaCl2) for 7 days. Collagen secretion and non-collagenous proteins secretion were evaluated using the Semi-Quantitative Tissue Total Collagen Staining Kit (Chondrex, Inc). Mean ± SEM of n=5. Statistical significance was calculated using two-way ANOVA. *p<0.05, **p<0.01 and ****p<0.0001 vs. same genetic background non-treated cells. & p<0.05, &&& p<0.001, &&&& p<0.0001 vs. 7 days treated with high Pi-Ca2+.

FIG 4. ROLE FOR HERPUD1 DURING CALCIFICATION



Proposal scheme to explain the results, considering the calcification triggers (high Pi), the VSMC osteo-conversion genetic response, the increase of gene expression, particularly of protein-quality control modulators and the increase in collagen and non-collagenous auxiliary proteins secretion to favor the ectopic mineralization of blood vessels.