Osteogenic differentiation of human lens epithelial cells might contribute to lens calcification

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Disclosures:

The authors have no conflicting financial interests.

ABSTRACT

Calcification of the human lens has been described in senile cataracts and in young patients with congenital cataract or chronic uveitis. Lens calcification is also a major complication of cataract surgery and plays a role in the opacification of intraocular lenses. A cell-mediated process has been suggested in the background of lens calcification, but so far the exact mechanism remained unexplored. Lens calcification shares remarkable similarities with vascular calcification; in both pathological processes hydroxyapatite accumulates in the soft tissue. Vascular calcification is a regulated, cell-mediated process in which vascular cells undergo osteogenic differentiation. Our objective was to investigate whether human lens epithelial cells (HuLECs) can undergo osteogenic transition *in vitro*, and whether this process contributes to lens calcification. We used inorganic phosphate (Pi) and Ca to stimulate osteogenic differentiation of HuLECs. Osteogenic stimuli (2.5 mmol/L Pi and 1.2 mmol/L Ca) induced extracellular matrix mineralization and Ca deposition in HuLECs with the critical involvement of active Pi uptake. Osteogenic stimuli almost doubled mRNA expressions of osteo-/chondrogenic transcription factors *Runx2* and *Sox9*, which was accompanied by a 1.9-fold increase in Runx2 and a 5.5-fold increase in Sox9 protein expressions. Osteogenic stimuli induced mRNA and protein expressions of alkaline phosphatase and osteocalcin in HuLEC. Ca content was higher in human cataractous lenses, compared to non-cataractous controls (n=10). Osteocalcin, an osteoblast-specific protein, was expressed in 2 out of 10 cataractous lenses. We conclude that osteogenic stimuli induce osteogenic differentiation of HuLECs and propose that this mechanism might play a role in lens calcification.

Key words: human lens epithelial cells, osteogenic differentiation, calcification, cataract, aging disease

1. INTRODUCTION

According to the Global Eye Health Statistics, at least 25 million eyes develop decreased visual acuity due to cataract formation annually. Currently, cataract is the leading cause of blindness worldwide [1]. The etiology of cataract is diverse, however, aging is the most common risk factor for cataract formation [2]. This is supported by the notion that about 25% of the population over 65 and 50% of the population over 80 is affected [3]. Currently, the only available treatment for cataract is surgical intervention [4].

By definition, a cataract is an opacity of the lens of the eye. A lot of effort has been made to determine the etiology of cataracts, and several relevant mechanisms have been identified. Lens proteins play a crucial role in maintaining the transparency of the lens. These proteins are extremely long-lived, as there is no protein synthesis and turnover in mature fiber cells. Consequently, age-related modifications of these proteins accumulate over a lifetime that can result in the formation of insoluble protein aggregates, which are present in cataractous lenses [5-7].

Lens epithelial cells (LECs) show phenotypic plasticity and therefore they can respond to environmental changes. For example, lens epithelial cells are able to undergo epithelial-to-mesenchymal transition (EMT) in response to injury or growth factors (*e.g.* transforming growth factor beta) [8]. LECs that undergo EMT show altered cell morphology, aberrant proliferation and migration properties, and are characterized by increased expression of extracellular matrix (ECM) proteins, intermediate filaments and various integrins as well as decreased expression of epithelial cell proteins [9]. EMT has been shown to play a major role in the formation of anterior subcapsular cataract, as well as in the opacification of intraocular lenses [10].

Determination of the chemical composition of senile cataractous lenses has revealed high concentrations of calcium (Ca) and phosphate, which form bone-like hydroxyapatite crystals [11-14]. Occasionally, calcification also occurs in young patients with congenital cataract or chronic uveitis [13, 15], while it can be a major complication of cataract surgery and play a role in the opacification of intraocular lenses [12, 16-18]. Although a cell-induced mechanism has been suggested in the background of lens calcification, no effort has been made to uncover more in depth this process [15, 19].

There is a remarkable overlap between the risk factors of cataract and vascular calcification, suggesting a common etiology of these diseases. Besides aging that largely contributes to both cataractogenesis and vascular calcification, diabetes and chronic kidney disease are also common predisposing factors for both diseases [20-23]. Nowadays, it is largely accepted that vascular calcification is a highly-regulated cell-mediated process, which involves osteo-/chondrogenic differentiation of vascular smooth muscle cells (VSMCs) [24-26]. Osteogenic triggers (*e.g.* inorganic phosphate (Pi), Ca, BMP-2 (bone-morphogenetic protein 2), glucose) initiate the osteo-/chondrogenic transition of VSMCs via the upregulation of osteo-/chondrogenic transcription factors, such as Runx2 (Runt-Related Transcription Factor 2), Sox9 ((Sex Determining Region Y)-Box 9) and Msx2 (Msh Homeobox 2) [25, 27-30]. Furthermore, calcifying VSMCs express alkaline phosphatase (ALP), an early marker of osteogenesis, and osteocalcin (OCN), a major non-collagenous protein in the bone matrix that regulates mineralization [30].

The aim of this study was to investigate whether human LECs (HuLECs) can undergo osteo-/chondrogenic differentiation *in vitro*. We revealed that osteogenic stimuli induced osteo-/chondrogenic phenotype transition of HuLECs via the upregulation of osteo-/chondrogenic transcription factors Runx2 and Sox9. Osteogenic stimuli upregulated ALP and OCN expressions and induced ECM mineralization. Furthermore, we observed that Ca accumulated in human cataractous lenses, when compared to non-cataractous control lenses, while some of the cataractous samples expressed the osteoblast-specific protein, OCN. We propose that osteo/chondrogenic differentiation of HuLECs might be involved in lens calcification.

2. **MATERIALS AND METHODS**

2.1. Cell culture and reagents

HuLECs were purchased from ScienCell Research Laboratories (San Diego, CA, USA). Cells were maintained in EpiCM supplemented with EpiCGS, 10% FBS, 10000 U/ml penicillin and 10000 μg/mL streptomycin (ScienceCell Research Laboratories). Cells were grown to confluence and used from passages 3 to 4. In certain experiments we used immortalized human lens epithelial cells (iHuLEC, CRL-11421) derived from ATCC (Manassas, VA, USA). Cells were cultured according to the manufacturer's instructions. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.2. Human lens specimens

Human cataractous lenses (n=10, M/F: 4/6, age: 71.5 ± 9.1) were collected from patients who had undergone cataract surgery (extracapsular cataract extraction) at the Department of Ophtamology, Faculty of Medicine, University of Szeged after patient informed consent was obtained. All cataracts were senile type including 5 mixed nuclear and cortical cataracts, 2 nuclear cataracts and 3 total/mature cataracts. Non-cataractous lenses (n=10, M/F: 3/7, age: 64.4 ± 11.5) were derived from human cadaver eyes removed within 24 hours of death at the Department of Pathology, Faculty of Medicine, University of Debrecen. All tissue collection complied with the Guidelines of the Helsinki Declaration and was approved by the Regional and Institutional Research Ethics Committee at the University of Debrecen, Hungary (DE OEC: 3094–2010). Lenses were snap-frozen in liquid nitrogen and stored at -70 °C until analysis. For Ca measurement, one-quarter of each lens was suspended in 300 μl HCl (0.6 mol/L) for 1 hour at room temperature. Samples were centrifuged (2000 *g*, 5 min, RT) and supernatants were obtained. For OCN measurements, the remained three-quarters of each lens was suspended in 300 μl EDTA (0.5 mol/L, pH 6.9), frozen and thawed twice and sonicated (continuous mode, 30 sec); supernatants were collected after a brief centrifugation (2000 *g*, 5 min, 4°C).

2.3. Induction of osteogenesis

Osteogenic differentiation was induced as described previously [31]. Growth medium was supplemented with inorganic phosphate (NaH_2PO_4-Na_2HPO_4, pH 7.4) and $CaCl_2$, as indicated on the individual graphs. Osteogenic medium was changed every other day. For time-course experiments, the

first day of culture in osteogenic medium was defined as Day 0. In some experiments we triggered osteogenic differentiation of HuLECs in the presence of phosphonoformic acid (PPFA, 1.5 mmol/L).

2.4. Determination of cell viability

Cell viability was determined by the MTT assay as previously described [32]. Briefly, cells were cultured and treated in 96-well plates for the indicated time. Then cells were washed with PBS, and 100 μL of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (0.5 mg/mL) solution in HBSS was added. After a 4-hour incubation the MTT solution was removed, formazan crystals were dissolved in 100 μL of DMSO and optical density was measured at 570 nm.

2.3. Alizarin Red staining

After washing with PBS, the cells were fixed in 4% paraformaldehyde and rinsed with deionized water thoroughly. Cells were stained with Alizarin Red S solution (2%, pH 4.2) for 20 minutes at room temperature. Excessive dye was removed by several washes in deionized water. Extracellular Ca deposition was stained in red color using Alizarin Red S dye.

2.4. Quantification of Ca deposition

Cells grown in 96-well plates were washed twice with PBS and decalcified with 0.6 mol/L HCl for 30 minutes. Ca content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur, Kampenhout Belgium) as previously described [33].

2.5. Quantitative reverse transcription-polymerase chain reaction

RNA was isolated from cells using Trizol (RNA-STAT60, Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's protocol. Two micrograms of RNA were reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Quantitative real-time PCR was performed using iTaq Universal Probes Supermix (BioRad Laboratories, Hercules, CA, USA) and predesigned primers and probes (TaqMan® Gene Expression Assays) to detect *Runx2* (Hs.535845), *Sox9* (Hs.01001343), *ALP* (Hs.00768162), *OCN* (Hs.01587814), *alpha smooth muscle actin* (*α-SMA*, Hs.00426835), *Slug* (Hs.001619), *E-cadherin* (Hs.1023894), *paired box protein 6* (*Pax-6*, Hs.00240871) and *hypoxanthine-guanine phosphoribosyl transferase* (*HPRT*, Hs.412707). Relative mRNA expressions were calculated with the ΔΔCt method using *HPRT* as internal control.

2.6. Western Blot analysis

To evaluate Runx2, Sox9 and ALP protein expressions, cell lysates were run on 10% SDS-PAGE. Western Blotting was performed with the use of a polyclonal anti-Runx2 antibody (Proteintech, Rosemont IL, USA) at 1:1000 dilution, anti-Sox9 antibody (Abcam, Cambridge, UK) at 1:1000 dilution, a polyclonal anti-ALP antibody at 1:200 dilution (Santa Cruz Biotechnology Inc., Dallas, TX, USA), followed by HRP-labeled anti rabbit IgG antibody (Amersham Biosciences Corp., Piscataway, NJ, USA). Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Amersham Biosciences Corp., Piscataway, NJ, USA). After detection, the membranes were stripped and reprobed for GAPDH using anti-GAPDH antibody at a dilution of 1:1000 (Novus Biologicals, Littleton, CO, USA). Results were quantified by using Alpha DigiDoc RT (Alpha Innotech, San Leandro, CA, USA) quantification system.

2.7. Quantification of OCN

For OCN detection, the ECM of cells grown on 6-well plates was dissolved in 100 μL of EDTA (0.5 mol/L, pH 6.9). OCN content of the EDTA-solubilized ECM samples was quantified by an enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA).

2.8. Phosphate measurement

After a 24-hour treatment, cells were washed twice with PBS and solubilized in a lysis buffer containing 10 mmol/L TrisHCl, 5 mmol/L EDTA, 150 mmol/L NaCl (pH 7.2), 1% Triton X-100, 0.5% Nonidet P-40 and protease inhibitors (Complete Mini, F. Hoffmann-La Roche Ltd., Basel, Switzerland). Phosphate levels of cell lysates were determined by the QuantiChrome phosphate Assay Kit (Gentaur, Kampenhout Belgium).

2.9. Statistical analysis

Data are shown as mean ± SD. Statistical analysis was performed by ANOVA test followed by post hoc Newmann-Keuls test for multiple comparisons. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1. Osteogenic stimuli induce ECM mineralization of HuLECs in a cell-mediated manner without affecting cell viability

Elevated levels of phosphate and Ca are well-characterized and pathophysiologically relevant triggers of VSMC mineralization and osteogenic differentiation. To determine whether osteogenic stimuli induce ECM mineralization in HuLECs, cells were cultured in calcification medium which was prepared by addition of Pi and Ca at the indicated concentrations (0-3.5 mmol/L and 0-1.2 mmol/L, respectively) to the growth medium. Granular deposits developed in HuLECs in a dose-dependent manner in 7 days, but not in the control cells (cultured in growth medium without Pi or Ca addition) as demonstrated by Alizarin Red staining (Fig 1A). Pi alone induced ECM mineralization of HuLECs at the dose of 3.5 mmol/L, whereas Ca alone could not trigger matrix mineralization. Ca synergized Pi-induced mineralization at each tested concentrations. To quantify the extent of matrix mineralization we measured Ca content of the ECM after culturing the cells for 7 days under osteogenic conditions. We found that Pi and Ca synergistically increased the Ca content of the ECM in HuLECs (Fig. 1B).

We made a time course experiment to demonstrate that calcification is an active cell-mediated process and not passive precipitation of hydroxyapatit. In this experiment we treated living or PFA-fixed HuLECs with osteogenic medium (addition of 2.5 mmol/L Pi and 1.2 mmol/L Ca to the growth medium). We determined the amount of Ca deposition on Day 0, 3, 5 and 7 (Fig. 1C). Ca deposition started on Day 5 in the living cell-containing wells and increased further until Day 7 (Fig. 1C). In contrast, we did not observe ECM mineralization in PFA-fixed cells (Fig. 1C).

Apoptotic cell death has been reported during calcification and matrix mineralization of VSMCs [34], therefore we examined whether matrix mineralization of HuLECs is associated with cell death. We cultured HuLECs in osteogenic medium for 7 days, and then we assessed cell viability by MTT assay. In contrast to VSMCs, we observed that osteogenic stimuli did not induce cell death in HuLECs (Fig. 1D). Overall, these results suggest that Pi-mediated HuLEC mineralization is a cell-mediated active process, in which cell death does not play a role.

3.2. Pi uptake is critically involved in osteogenic stimuli-triggered calcification of ECM in HuLECs

Active transport of phosphate into the cells via type III sodium-dependent phosphate cotransporters is absolutely required for phosphate-induced osteogenic differentiation and calcification of VSMCs [35]. Phosphate treatment increases intracellular phosphate levels in VSMCs [31], and inhibition of phosphate transport by PPFA prevents calcification of VSMCs [36] therefore next we asked whether the same occurs in HuLECs. We found that a 24-hour phosphate treatment dose-dependently increased intracellular phosphate levels in HuLECs, which increase was completely abolished in the presence of PPFA (Fig. 2A), suggesting that active phosphate transport takes place in these cells, similarly to VSMCs. Then we investigated whether inhibition of phosphate uptake influence mineralization of HuLECs. We treated HuLECs with osteogenic medium (0.6 mmol/L Ca, 0; 2.5; 3.0; 3.5 mmol/L Pi) in the presence or absence of PPFA for 7 days and accessed ECM mineralization by Alizarin Red staining. As shown by Fig 2B, PPFA completely inhibited ECM mineralization in HuLECs. Quantification of the Ca content of ECM supported the results obtained by Alizarin Red staining (Fig. 2C). We concluded that active transport of phosphate is critical in phosphate-triggered ECM mineralization in HuLECs.

3.3. HuLECs mineralization is accompanied by upregulation of osteo-/chondrogenic transcription factors: Runx2 and Sox9

VSMC differentiation to an osteogenic phenotype, which is characterized by elevated levels of osteogenic and/or chondorgenic transcription factors, has been identified as the underlying mechanism of vascular calcification [30, 37]. Therefore we assessed the expression of the key osteogenic transcription factor, Runx2, and the master regulator of chondrogenesis, Sox9, to examine whether osteogenic differentiation occurs in HuLECs upon osteogenic stimuli. We exposed HuLECs to osteogenic medium (2.5 mmol/L Pi, 1.2 mmol/L Ca) for 24 hours then measured mRNA levels of Runx2 and Sox9. The osteogenic stimuli almost doubled the *Runx2* and *Sox9* mRNA levels in HuLECs compared to control cells (Fig. 3A and B). Western blot analysis confirmed the gene expression results and revealed that the osteogenic stimuli can trigger a 1.9-fold increase in Runx2 (Fig. 3C and E) and a 5.5-fold elevation in Sox9 (Fig 3D and F) protein expressions. These results suggest that Pi and Ca induce osteo-/chondrogenic differentiation of HuLECs.

3.4. ALP and OCN are upregulated in mineralizing HuLECs

ALP, being a phosphohydrolytic enzyme plays an important role in bone mineralization as well as in pathological mineralization in non-osteogenic tissues [38]. First we determined ALP mRNA levels at basal (control) and calcifying conditions (Pi/Ca) in HuLECs (Fig. 4A). Osteogenic treatment (7 days, 2.5 mmol/L Pi and 1.2 mmol/L Ca) resulted in a ~ 1.7-fold increase in *ALP* mRNA (Fig. 4A). This increase in *ALP* mRNA level was associated with increased protein expression of ALP (Fig. 4B). Both Pi (3 mmol/L) and Ca (1.2 mmol/L), when applied separately, triggered a mild (~ 1.7-fold) increase in ALP expression in HuLECs (Fig. 4B and C). When Pi (2 mmol/L) and Ca (1.2 mmol/L) were applied together, we obtained a 3-fold increase in ALP expression, whereas 3 mmol/L Pi together with 1.2 mmol/L Ca resulted in a 4.1-fold increase in ALP level (Fig. 4B and C). These results suggest that Pi and Ca act in a synergistic manner in increasing ALP expression in HuLECs (Fig. 4B and C). Lastly, we assessed ALP activity, and found that both Pi (3 mmol/L) and Ca (1.2 mmol/L) caused a mild elevation in ALP activity when applied separately, and they acted in a synergistic way to further increase ALP activity, when applied together (Fig. 4D).

OCN, being a major non-collagenous protein of fully mineralized bone-matrix, is highly expressed in calcifying VSMCs [37, 39]. Therefore next we checked whether OCN gets also upregulated in HuLECs under osteogenic stimulation. We cultured HuLECs under control, and calcifying conditions (Pi/Ca) for 7 days, and measured *OCN* mRNA levels. Osteogenic stimuli induced a more than 2-fold elevation in *OCN* mRNA levels (Fig. 5A). Then the OCN content of the ECM of HuLECs cultured in the presence or absence of Pi and Ca for 7 days was analyzed by ELISA. No OCN could be detected in the EDTA-solubilized ECM of HuLECs cultured under basal conditions (without Pi or Ca supplementation) (Fig. 5B). Supplementation of the growth medium with Pi (3 mmol/L) or Ca (1.2 mmol/L) caused a minor increase in OCN levels resulting 0.156 ng/ml and 1.027 ng/ml OCN respectively (Fig. 5B). Importantly, when Pi and Ca were applied together, a marked elevation in OCN levels was observed in the ECM of HuLECs (Fig. 5B). These findings further support our notion that Pi and Ca trigger osteogenic differentiation of HuLECs in a synergistic manner.

3.5. Osteogenic differentiation of HuLECs shares some similarities with EMT

Certain stimuli can induce EMT in lens epithelial cells. To see whether HuLECs are undergo EMT upon osteogenic stimulation, we treated the cells with osteogenic medium (Pi: 2.5 mmol/L, Ca: 1.2 mmol/L) and measured mRNA levels of α-SMA, Slug, Pax-6 and E-cadherin on Day 1 and Day 5. We found that osteogenic stimulation decreased *α-SMA*, and *E-cadherin* mRNA levels (Fig. 6 A and D). Expression of *Slug* mRNA increased in a time-dependent manner, reaching about 2.5-fold elevation on Day 5 (Fig. 6B). We observed a slight decrease in *Pax-6* mRNA level on Day1 which was normalized by Day 5 (Fig. 5C). We concluded that osteogenic differentiation shares some similarities with EMT.

3.6. Osteogenic stimuli induce osteogenic differentiation and ECM mineralization in iHuLECs

Because we performed all of the differentiation experiments with the use of primary cells derived from one particular donor, we wanted to see whether well-characterized lens epithelial cells behave a similar manner regarding osteogenic differentiation. Therefore we exposed iHuLEC to calcification medium which was prepared by addition of Pi and Ca at the indicated concentrations (0-3.5 mmol/L and 0-1.2 mmol/L, respectively) to the growth medium. ECM mineralization was assessed by Alizarin Red staining (Fig. 6A) on Day 7. Pi and Ca triggered ECM mineralization of iHuLEC cells similarly to that of primary HuLEC cells. We quantified the extent of matrix mineralization by measuring Ca content of the ECM after culturing the cells for 7 days under osteogenic conditions. As shown in Fig. 6B, Pi and Ca synergistically increased Ca content of the ECM in iHuLECs. We addressed whether osteogenic stimulation increases the expression of the osteogenic transcription factor Runx2, and its target protein OCN. We exposed HuLECs to osteogenic medium (2.5 mmol/L Pi, 1.2 mmol/L Ca) for 24 hours then measured mRNA levels of *Runx2* and *OCN*. Osteogenic stimuli triggered a 3.5 ± 0.6-fold increase in *Runx2* and a 1.8 ± 0.1-fold elevation in *OCN* mRNA levels in iHuLECs compared to controls (Fig. 6C and D).

3.7. Presence of Ca and OCN in human cataractous lenses

We aimed to explore whether osteogenic differentiation of HuLECs plays a role in human cataract formation. We analyzed Ca and OCN content of human non-cataractous lenses (n=10) derived from cadaver human eyes and cataractous lenses (n=10) collected from patients who had undergone cataract surgery. The Ca content of cataractous lenses was significantly higher than that of control lenses (0.123 ± 0.097 vs. 0.019 ± 0.016 μg Ca/mg tissue) (Fig. 6). OCN was non-detectable in non-

cataractous lenses. In contrast, we found low but measurable amounts of OCN in 2 out of the 10 cataractous lenses (0.52 ng/ml and 0.69 ng/ml). Interestingly, OCN positivity was associated with the highest Ca levels (above 0.25 μg Ca/mg tissue). These results suggest that osteogenic differentiation might play a role in lens calcification *in vivo*.

4. DISCUSSION

In this study we examined the process of osteo-/chondrogenic differentiation of HuLECs *in vitro* and the potential involvement of this differentiation process in lens calcification. Osteo-/chondrogenic differentiation is essential for the growth, repair and remodeling of bone and cartilage tissues, therefore it has an important physiologic role. On the other hand, pathological osteo-/chondrogenic differentiation is involved in ectopic calcification, when precipitation and deposition of Ca-phosphate salts occurs in the form of hydroxyapatite in extra-skeletal soft tissues. Although ectopic calcification has long been considered as a passive and degenerative process, nowadays it is recognized as a cell-mediated, highly regulated pathway, which shares many features with bone formation. The most frequently seen and the most studied form of ectopic calcification is vascular calcification, in which a subpopulation of vascular cells, called calcifying vascular cells, undergo osteogenic differentiation in response to an osteogenic stimuli [40]. These osteoprogenitor cells in the vasculature can derive from different cell types including mesenchymal stem cells, monocytes, pericytes and VSMCs [41].

Both vascular calcification and cataract formation are strongly associated with aging, diabetes and chronic kidney disease [20-23]. Moreover, recent studies revealed that mutations of certain genes lead to the appearance of cataract and diverse types of ectopic calcification. For example, a homozygous mutation in the lamin A/C gene has been described in a 44-year-old patient, who suffered from tendon calcification and cataract [42]. Activating mutation of the Ca-sensing receptor was also found to be associated with cataract formation and ectopic calcification in a mouse model [43]. Additionally, cataract and calcification of the basal ganglia has been described in a patient who suffered from progeroid syndrome - a rare genetic disorder that mimics physiological aging, making the affected individuals appear older than what they are [44]. Furthermore, in a recent study of 6 patients suffering from Cockayne syndrome, the co-presence of intracranial calcification and cataract, was reported [45]. These results suggest that ectopic calcification and cataract might have common etiological factors.

Osteo-/chondrogenic differentiation of VSMCs is one of the critical cellular events in vascular calcification. It is long recognized that disturbances of the phosphate and Ca metabolism, that results hypercalcemia and hyperphosphatemia, play a major role in vascular and other soft tissue calcification

in patients with chronic kidney disease [46]. In parallel with the clinical findings, elevated Pi and Ca induce osteogenic differentiation and ECM mineralization of VSMCs *in vitro* [27]. Pi-induced mineralization of VSMCs became an important model to study the mechanism of vascular calcification. We used this model, and stimulated HuLECs with Pi and Ca, to test whether osteo-/chondorogenic differentiation and ECM mineralization can be triggered in these cells *in vitro*. We found that elevated Pi induces matrix mineralization of HuLECs, and Ca synergizes the effect of Pi.

Although there is a case report suggesting a pathological role of elevated Pi in cataract formation [47], it is an open question whether elevated Pi and Ca levels could be clinically relevant in lens calcification. Lens is avascular tissue nurtured by aqueous humor. There is a single article in which the authors investigated Ca and Pi content of aqueous humor in cataract patients [48]. According to that study Ca concentration of aqueous humor is 5.74 mg/dL, and the Pi is 2.19 mg/dL in non-diabetic subjects with cataract. The osteogenic medium we used to trigger osteogenic differentiation of HuLECs contained twice as much Ca and five-times more Pi than the aqueous humor of cataract patient, but we assume that osteogenic differentiation of HuLECs is a multifactorial process *in vivo*. Imbalance of calcification inducers and inhibitors is behind vascular calcification. Presumably that could be the case in lens calcification in which different inducers – not yet characterized – could act in a synergistic manner to induce osteogenic differentiation of lens cells. What regards calcification inhibitors, mass spectrometry analysis revealed the presence of alpha-2-HS-glycoprotein (fetuin-A), the well-known inhibitor of vascular calcification, in aqueous humor [49]. Further work needs to be done to identify the clinically relevant inducers and inhibitors of lens calcification.

Phosphate-induced osteogenic differentiation and calcification of VSMCs require active transport of extracellular phosphate into the cells [36] that is accomplished by type III sodium-dependent phosphate cotransporters PiT-1 and PiT-2 [35]. Recently, the critical involvement of PiT-1 in chondrogenesis has been demonstrated as well [50]. Currently, we lack information about phosphate handling of HuLECs and the expression of such type III sodium-dependent phosphate cotransporters. Nevertheless, we showed that phosphate treatment increased intracellular phosphate levels in HuLECs, similarly to that of VSMCs, which suggests the presence of active phosphate

transport in lens epithelia. Moreover, we found that inhibition of phosphate uptake by PPFA results in diminished mineralization of ECM. Considering the critical involvement of phosphate uptake in the process of ECM mineralization, further research needs to be done to identify the transporter(s) involved in phosphate uptake and subsequent mineralization of LECs.

Runx2 and Sox9, the key transcription factors regulating osteoblastogenesis and chondrogenesis, respectively, play a role in vascular calcification as well. This notion is strongly supported by the observation that these osteo-/chondrogenic transcription factors are highly expressed in calcified human arterial samples compared to noncalcified vessels [30]. Furthermore, *in vitro* studies revealed that Runx2 and Sox9 are upregulated upon osteo-/chondrogenic transition of VSMCs [25, 51, 52]. Here, we showed that HuLECs express both Runx2 and Sox9 under basal conditions. Production of Runx2 and/or Sox9 is not a unique feature of LECs, as epithelial cells of different origin such as amniotic, proximal tubular, dental and mammary epithelial cells also express Runx2 and/or Sox9 [53-57]. Elevated expressions of Runx2 and/or Sox9 in these epithelial cells of diverse origin have been linked to a stem-cell like phenotype with osteogenic differentiation and/or regenerative potentials [53-57]. The osteogenic stimuli implemented in the present study upregulated the Runx2 and Sox9 expression, suggesting that osteo-/chondrogenic differentiation of HuLECs occurs *in vitro*.

Tissue-nonspecific ALP enzymes (TNAP) are ubiquitously expressed in all tissues, with the highest production in liver, kidney and bone. TNAP plays a critical role in bone mineralization, as well as in ectopic calcification [38]. The effect of TNAP relies on its ability to hydrolyze extracellular pyrophosphate, a potent inhibitor of hydroxyapatite formation [58]. Overexpression of TNAP in VSMCs in mice results in extensive vascular calcification, high blood pressure, cardiac hypertrophy, and early death, whereas pharmacological inhibition of TNAP activity reverses these deleterious effects [59]. Interestingly, ALP activity is significantly elevated in the plasma of cataract patients [60]. Since increased ALP has been associated with elevated γ-glutamyl transferase level, it was suggested that subclinical liver dysfunction is a risk factor for cataract formation [60], but this idea was not strengthened by further studies. Besides hepatobiliary disorders, elevation of ALP is also detected in bone diseases associated with increased osteoblastic activity and bone remodeling, such as Paget's disease, hyperparathyroidism, rickets and osteomalacia, fractures, and malignant tumors. Accelerated

bone growth in children and juveniles can also cause a rise in ALP activity. *In vitro* studies revealed that ALP expression and activity are highly elevated in calcifying VSMCs [61]. Similarly to that of VSMCs, we observed that mineralization of HuLECs is accompanied by increased expression and enzymatic activity of ALP. Further studies are needed to explore whether vascular calcification or other types of ectopic calcification are associated with systemic elevation of ALP activity.

OCN is highly expressed in the fully mineralized bone-matrix, cartilage and dental tissues. Besides its long-known function of binding hydroxyapatite in osteochondral tissues, OCN is currently considered as a regulatory molecule of mineralization and a marker of bone metabolism. Recent findings also highlighted new extra-skeletal roles for OCN, in which OCN acts as a hormone [62]. High OCN expressions have been found in mineralized extra-skeletal tissues such as calcified vessels and skin lesions in patients with calcific uremic arteriolopathy [30, 63]. Recently, a correlation between calcifying OCN-positive endothelial progenitor cells and spotty calcification in patients with coronary artery disease was described [64]. These data suggest that OCN plays a role in ectopic calcification. Under *in vitro* conditions calcifying VSMCs highly express OCN [37, 39], and here we revealed that osteogenic stimuli upregulate OCN expression in HuLECs as well. Importantly, we showed that OCN is expressed in some human cataractous lenses, but not in control lenses. Overall, our findings strongly suggest that osteogenic stimuli represented by elevated Pi and Ca induce osteo-/chondrogenic differentiation of HuLECs with the critical involvement of phosphate uptake via the upregulation of osteo-/chondrogenic transcription factors Runx2 and Sox9. The resulting osteoblast-like cells express osteoblast-specific proteins, such as ALP and OCN and exhibit ECM mineralization. We propose that this mechanism might contribute to lens calcification, which is supported by our finding that some human cataractous lenses express the osteoblast-specific protein, OCN.

5. ACKNOWLEDGMENTS

This work was supported by a Hungarian Government grant OTKA-K116024 and the University of Debrecen Research Grant RH/751-2/2015. V.J. was co-financed by the European Union and the European Social Fund TÁMOP-4.2.2.A/2-11-1-2012-0001. GP and the Stem Cells and Eye Research Laboratory, Department of Ophthalmology, Faculty of Medicine, University of Szeged, Hungary, have been supported by the National Brain Research Program (KTIA_NAP_13-A_III/9).

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7. FIGURE LEGENDS

Figure 1

Osteogenic stimuli induce matrix mineralization and Ca deposition of HuLECs in a cell-mediated manner, without affecting cell viability

(A, B and D) HuLECs were cultured in growth medium supplemented with Pi (0-3.5 mmol/L) and Ca (0-1.2 mmol/L) as indicated for 7 days. (A) Ca deposition as a readout of ECM mineralization was visualized by Alizarin Red staining. Representative image of stained plates from three independent experiments is shown. (B) Ca content of the HCl-solubilized ECM is presented. Data are expressed as mean ± SD of three independent experiments performed in quadruplicates. (C) Living (closed circles) or PFA-fixed HuLECs (open circles) were treated with osteogenic medium (2.5 mmol/L Pi and 1.2 mmol/L Ca). ECM Ca content was determined on Day 0, 3, 5 and 7. Results are presented as mean ± SD of two independent experiments performed in quadruplicates. (D) After 7 days of treatment with osteogenic medium cell viability was determined by MTT assay. Data are expressed as mean ± SD of three independent experiments performed in quadruplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

Figure 2

Pi uptake is critically involved in osteogenic stimuli-triggered calcification of ECM in HuLECs

(A-C) HuLECs were cultured in growth medium or osteogenic medium supplemented with Pi (2.5, 3.0, 3.5 mmol/L) and Ca (0.6 mmol/L) in the presence or absence of PPFA (1.5 mmol/L). (A) After a 24-hour treatment intracellular Pi levels were determined from the cell lysates. Results are presented as mean ± SD of three independent experiments performed in triplicates. (B) Ca deposition as a readout of ECM mineralization was visualized by Alizarin Red staining on Day 7. Representative image of stained plates from two independent experiments is shown. (C) Ca content of the HCl-solubilized ECM was measured on Day 7. Data are expressed as mean ± SD of two independent experiments performed in quadruplicates. ***$p < 0.005$

Figure 3

Osteogenic stimuli trigger upregulation of osteo-/chondrogenic transcription factors: Runx2 and Sox9

(A-F) HuLECs were cultured in growth medium or osteogenic medium (supplemented with 2.5 mmol/L Pi and 1.2 mmol/L Ca) for 24 hours. (A and B) Relative expressions of *Runx2* and *Sox9*

normalized to *HPRT* are presented. Results are presented as mean ± SD of three independent experiments performed in triplicates. (C and D) Representative Western blots from three independent experiments are shown. (E and F) Relative expressions of Runx2 and Sox9 normalized to GAPDH were determined by densitometric analysis. Graph shows mean ± SD of three separate experiments. $^{**}p < 0.01$, $^{***}p < 0.005$

Figure 4

Osteogenic stimuli increase ALP mRNA levels, protein expression and activity in HuLECs

(A) HuLECs were cultured in growth medium or osteogenic medium (supplemented with 2.5 mmol/L Pi and 1.2 mmol/L Ca) for 7 days. Relative mRNA level of *ALP* normalized to *HPRT* was determined by quantitative RT-PCR. Results are expressed as mean ± SD of three independent experiments performed in triplicates. (B-D) HuLECs were cultured in growth medium or osteogenic medium (supplemented with Pi and Ca as indicated) for 7 days and lysed. (B) Representative Western blots showing ALP and GAPDH expressions from three independent experiments are presented. (C) Relative expressions of ALP normalized to GAPDH were determined by densitometric analysis. Graph shows mean ± SD of three independent experiments. (D) ALP activities were determined from cell lysates and expressed as fold increase compared to control cells. Results are expressed as mean ± SD of three independent experiments performed in triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

Figure 5

Osteogenic stimuli increase OCN mRNA and protein expressions in HuLECs

(A) HuLECs were cultured in growth medium or osteogenic medium (supplemented with 2.5 mmol/L Pi and 1.2 mmol/L Ca) for 7 days. Relative mRNA level of *OCN* normalized to *HPRT* was determined by quantitative RT-PCR. Results are expressed as mean ± SD of three independent experiments performed in triplicates. (B) HuLECs were cultured in growth medium or osteogenic medium (supplemented with Pi and Ca, as indicated) for 7 days. ECM was solubilized in EDTA and OCN was determined by ELISA. Results are expressed as mean ± SD of three independent experiments performed in duplicates. $^{***}p < 0.005$

Figure 6.

Effect of osteogenic stimuli on markers of EMT

(A-D) HuLECs were cultured in osteogenic medium (supplemented with 2.5 mmol/L Pi and 1.2 mmol/L Ca) for 1 or 5 days. Relative mRNA levels of *α-SMA, Slug, Pax-6* and *E-cadherin* normalized to *HPRT* were determined by quantitative RT-PCR and expressed as % of non-stimulated controls. Results are presented as mean ± SD of two independent experiments performed in triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

Figure 7

Osteogenic stimuli induce ECM mineralization and osteogenic differentiation of iHuLECs

(A-B) iHuLECs were cultured in growth medium supplemented with Pi (0-3.5 mmol/L) and Ca (0-1.2 mmol/L) as indicated for 7 days. (A) Representative Alizarin Red staining from two independent experiments is shown. (B) Ca content of the HCl-solubilized ECM is presented. Data are expressed as mean ± SD of two independent experiments performed in triplicates. (C-D) iHuLECs were exposed to osteogenic medium (2.5 mmol/L Pi and 1.2 mmol/L Ca) for 24 hours. Relative mRNA levels of *Runx2* and *OCN* normalized to *HPRT* were determined by quantitative RT-PCR. Results are expressed as mean ± SD of three independent experiments performed in triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

Figure 8

Ca concentration is increased in human cataractous lenses

Ca levels were determined from human cataractous (n=10) and control (n=10) lenses. Results are expressed as μg/mg wet tissue.

8. ABBREVIATIONS

ALP: alkaline phosphatase

α-SMA: alpha-smooth muscle actin

BMP-2: bone-morphogenetic protein 2

Ca: calcium

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

EMT: epithelial-to- mesenchymal transition

HPRT: hypoxanthine-guanine phosphoribosyl transferase

HuLECs: human lens epithelial cells

iHuLEC: immortalized human lens epithelial cells

LEC: lens epithelial cells

Msx2: Msh Homeobox 2

OCN: osteocalcin

Pax-6: paired box protein

Pi: inorganic phosphate

PFA: paraformaldehyde

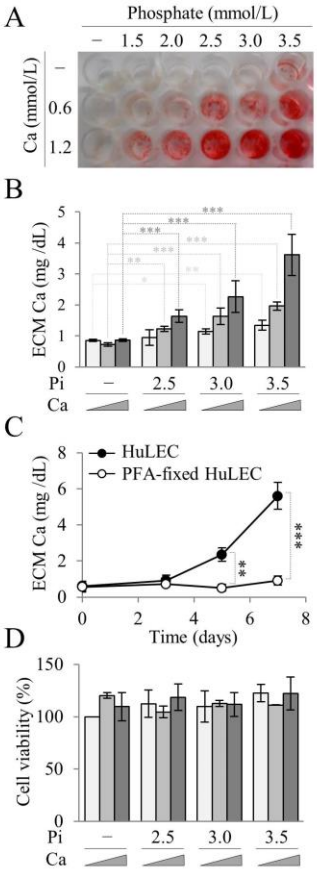
PPFA: phosphonoformic acid

Runx2: Runt-Related Transcription Factor 2

Sox9: (Sex Determining Region Y)-Box 9

TNAP: tissue-nonspecific alkaline phosphatase

VSMCs: vascular smooth muscle cells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Ca (mmol/L)
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1.2
B
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Pi
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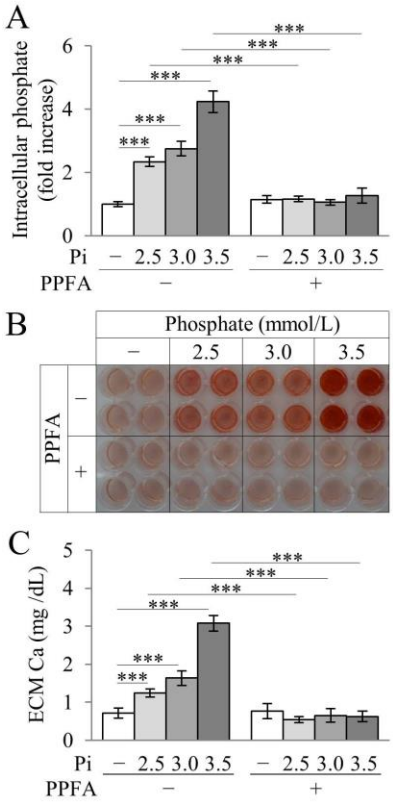
HuLEC
PFA-fixed HuLEC
ECM Ca (mg /dL)
Time (days)
D
Cell viability (%)
Pi
Ca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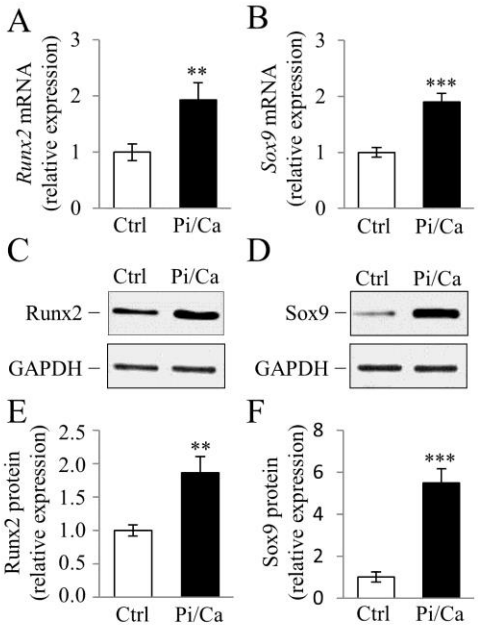
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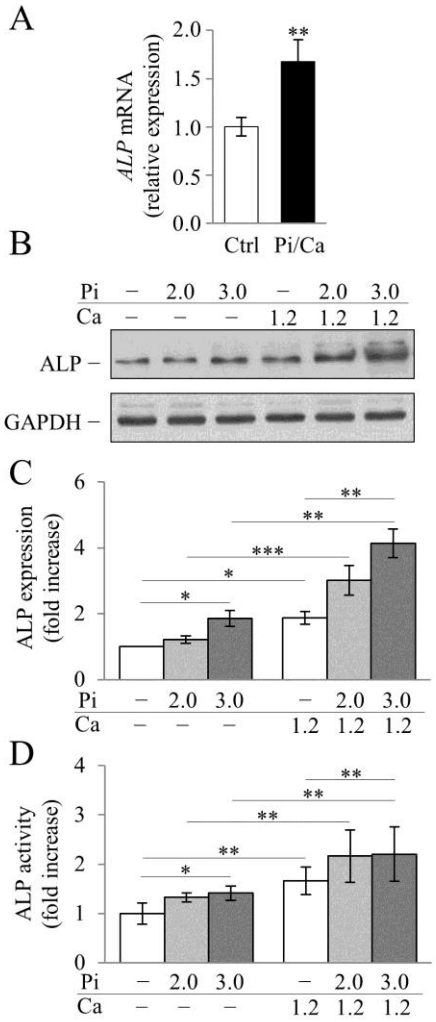
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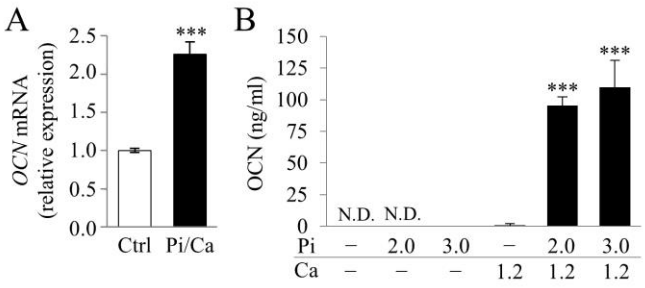
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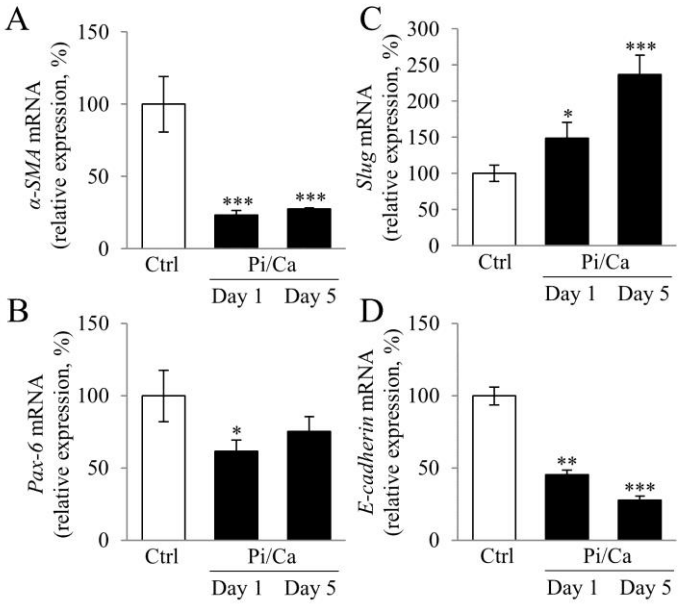
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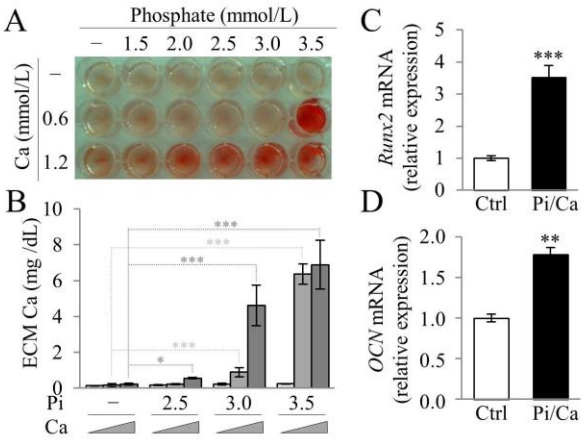
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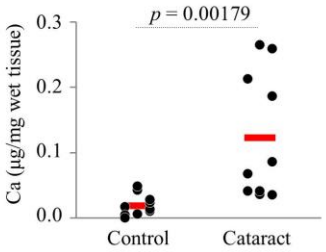
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