Short Thesis for the degree of doctor of philosophy (PhD)

Osteogenic differentiation of human mesenchymal stem cells and lens epithelial cells

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The examination takes place at the Department of Pediatric Hematology-Oncology, University of Debrecen at 11 am, on Oct 3, 2018

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The PhD Defense takes place at the Lecture Hall of ”A” Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1 pm, on Oct 3, 2018.

1. Introduction and literature review

Osteogenic differentiation plays a role in both physiological and pathophysiological processes. Osteogenic differentiation is essential in the process of mineralized tissue formation as well as in bone remodeling.. On the other hand pathophysiological osteogenic differentiation can be related to soft tissue mineralization.

Osteogenic differentiation of stem cells is a complex process, resulting in mature osteoblasts that are involved in the production of bone tissue. Inhibition of this process results in decreased anatomical and structural integrity of the bone tissue. It has long been known that chronic iron overload is associated with bone loss, and different mechanisms were identifiend behind this phenomenon.

Mineralization of soft tissues or so called ectopic calcification results in deposition of hydroxyapatite crystals similar to bone matrix in extraskeletal tissues. The most well-known type of ectopic calcification is vascular calcification, in which process osteogenic trans-differentiation of vascular smooth muscle cells has been established.

Interestingly, a substance similar to hydroxyapatite (HA) crystals found in calcified vessel walls present in cataractous lenses and in aqueous humour. The current mechanisms of cataract formation do not provide explanation for the presence of HA crystals in the cataractous lenses.

1.1. The importance of osteogenic differentiation in bone formation

1.1.1. Mesenchymal stem cells

Mesenchymal stem cells are multipotent stromal cells with the capability to differentiate into various mesenchymal lineages including but not limited to osteoblasts, chondrocytes and adipocytes. MSCs are self-renewable cells with high division and migration potential. The presence of MSCs was first observed in the bone marrow and subsequently isolated from a number of neonatal and adult tissues such as umbilical cord, placenta, fatty tissue, liver, spleen, skin, and lungs.

MSCs are spindle-shaped, adhesive cells that form monolayers and retain their ability to differentiate into various lineages including osteoblasts, adipocytes and chondrocytes when cultured *in vitro*.

1.1.2. The commitment of MSCs and the ability of multi-directional differentiation

In addition to the above-mentioned osteoblast, chondrocyte and adipocyte lineages, MSCs can also differentiate into other cell types, such as cardiac, skeletal or smooth muscle cells or fibroblasts. Specific master transcription factors control the engagement and differentiation of MSCs into the various lineages. In line with this notion, runt-related transcription factor 2 (Runx2) controls osteogenic, Sox9 drives chondrogenic and proliferator-activated receptor gamma regulates adipogenic differentiation of MSCs.

1.1.3. Osteogenic differentiation of MSCs.

Osteogenic differentiation of MSCs is driven by many secreted growth and differentiation factors and pathways, including transforming growth factor-beta 1, fibroblast growth factor, bone morphogenetic protein, wingless proteins, Indian hedgehog, which all converge on the master osteogenic transcription factor, Runx2. The crucial importance of Runx2 in osteogenic differentiation is highlighted by the finding, that Runx2 deficient mice completely lack differentiated osteoblasts resulting impaired bone formation, and die shortly after birth.

1.1.4. The role of MSCs in osteoporosis

Osteoporosis is a systemic bone metabolic disease that is highly prevalent in the elderly population, particularly in postmenopausal women, which results in increased susceptibility to fractures. Osteoporosis is currently one of the most significant health problems in the world, which contribute to 9 million osteoporosis-related fractures per year globally. In Hungary approximately 600,000 women and 300,000 men live with osteoporosis.

1.2. Relationship between osteoporosis and iron overload

1.2.1. Clinical observations

1.2.1.1. Thalassemia

Thalassemias are inherited blood disorders due to diverse mutations in the genes encoding hemoglobin chains causing various degrees of anemia. Severity of thalassemia varies widely from mild anemia (thalassemia intermedia) through severe forms (thalassemia major) to intrauterine death. Thalassemia major patients are on regular blood transfusion therapy in order to maintain sufficient Hb levels. The human body lacks active mechanism to excrete excess iron, therefore repeated blood transfusions lead to iron overload in these patients. Excess iron can deposit in body organs in particularly in pancreas, liver, pituitary and the heart. Thalassemia-associated osteoporosis and subsequent bone fractures remained to be one of the most frequent co-morbidity in thalassemia patients. Importantly, fracture risk correlates with severity of anemia and the frequency of blood transfusion in patients with thalassemia.

1.2.1.2. Sickle Cell Disease

Sickle cell disease (SCD) is an inherited autosomal recessive disorder, caused by a single nucleotide mutation in the Hb β-chain coding gene. SCD is one of the most prevalent genetic blood disorders worldwide affecting more than 300.000 annual births. SCD is associated with significant morbidity and shortened life span. Bone involvement is very common in SCD and can manifest in diverse ways including but not limited to bone and joint pain, vertebral bone deformities, osteopenia and osteoporosis and pathological fractures

1.2.1.3. Hemochromatosis

Hemochromatosis refers to a group of inherited disorders characterized by increased dietary iron uptake, which in some cases can lead to severe tissue iron overload. The accumulation of excess iron in the body tissues, damages different organs, particularly the liver, the pancreas and the heart. Several reports described

low bone density, osteoporosis and/or osteopenia in patients with different forms of hemochromatosis.

1.2.1.4. Menopausa

Postmenopausal osteoporosis is a systemic bone metabolism disorder affecting 30% of women over the age of 50. The disease is characterized by progressive bone loss and subsequent increase in the risk of fractures. Estrogen deficiency, a prominent feature of menopause, has been considered as the main cause of menopausal symptoms and disorders, but we have to note that menopausal transition is a complex process, in which besides hormonal shifts, iron metabolism is also altered. Parallel with the decline in estrogen level, a two- to threefold increase in serum ferritin concentration was detected in postmenopausal women.

1.3. Osteogenic differentiation in ectopic calcification

In ectopic calcification HA crystal deposition occurs in extraskeletal 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 process, 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. 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.

1.3.1. Ectopic calcification in the eye

Ectopic calcification of the eye has diverse manifestations and sometimes appears as part of an ectopic calcification disorder affecting other organs. HA crystals can

be found in cataractous lenses, in the anterior chamber and the cornea, as well as in the elastic layer of Bruch's membrane.

1.3.2. Epidemiology of the cataract formation

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

1.3.3. The mechanism of cataract formation

Epidemiological and experimental studies have revealed that exposure to UV can induce cataractogenesis. High glucose-induced apoptosis in human lens epithelial cells (LECs) is an initiating and key event in non-congenital cataracts. LECs show phenotypic plasticity and therefore they can respond to environmental changes. For example, LECs are able to undergo epithelial-to-mesenchymal transition (EMT) in response to injury or growth factors (e.g. transforming growth factor beta). 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. 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. 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. Oxidative stress has been identified as one of the major causes of age-related

diseases such as cataract, therefore inside the eye, oxidation processes can damage the proteins and lipids and they can contribute to cataract formation.

2. Aims

The aims of my scientific research were the followings:

1. Hypothesis: Iron overload inhibits osteogenic differentiation of mesenchymal stem cells.

1.1. To investigate the effect of iron on osteogenic differentiation and ECM mineralization in human bone marrow MSCs (BMSCs) *in vitro.*

1.2. To investigate the effect of ferritin on osteogenic differentiation and ECM mineralization in BMSCs.

1.3. We aimed to investigate the effect of iron overload on osteogenic differentiation in compact bone osteoprogenitor cells.

1.4. To analyze whether iron overload influences the adipogenic and chondrogenic differentiation of BMSCs.

2. Hypothesis: In response to osteogenic stimuli lens epithelial cells can undergo osteogenic differentiation and ECM mineralization, which can contribute to the formation of HA crystals in the cataractous lenses.

2.1. To investigate whether osteogenic stimuli induces osteogenic differentiation and ECM mineralization in human lens epithelial cells (HuLECs) *in vitro*.

2.2. To analyze differences and similarities between osteogenic differentiation and EMT in HuLECs.

2.3. Further aim was to investigate the presence of osteogenic markers in cataractous lenses.

3. Materials and methods

3.1. Cell culture

Human BMSCs and HuLECs were derived from ScienceCell and propagated according to the provided protocols.

3.2. Induction of osteogenic differentiation

At confluence, cells were switched to osteogenic medium which was prepared by adding inorganic phosphate (Pi) (0–3,5 mmol/L) and Ca^{2+} (0–1.2 mmol/L) to the growth medium.

3.3. Induction of chondrogenic and adipogenic differentiation

To induce chondrogenic and adipogenic differentiation, BMSCs were cultured in chondrogenic and adipocyte differentiation medium.

3.4. Alizarin Red staining

After washing with PBS, the cells were fixed in 4% paraformaldehyde. Cells were stained with Alizarin Red S solution (2%, pH 4.2) for 10 min at room temperature. Extracellular Ca deposition was stained in red color using Alizarin Red S dye.

3.5. Alcian blue staining

Alcian blue staining was performed with NovaUltra Special Stain Kit according to the manufacturer's instructions.

3.6. Oil Red O staining

After washing with PBS, the cells were fixed in 4% paraformaldehyde. Isopropanol (60%) was added to the cells for 5 min, followed by the addition of Oil Red Oworking solution (0.18% in 60% isopropanol) for 5 min. Excessive dye was removed by several washes in water.

3.7. Quantification of Ca deposition

Cells grown on 96-well plates were washed twice with PBS, and decalcified with HCl (0.6 mol/L) for 30 min at room temperature. Ca content of the HCl supernatants was determined by QuantiChrome Calcium Assay Kit.

3.8. Cell viability assay

Cell viability was determined by the MTT assay as previously described.

3.9. 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).

3.10. Measurement of ALP activity

Cells were lysed and 35 μL cell lysate was mixed with 130 μL of ALP Yellow Liquid Substrate. Formation of p-nitrophenol was followed at 405 nm for 30 min at 37 °C. Maximum slope of the kinetic curves was used for calculation of ALP activity which was then normalized to protein content.

3.11. Quantitative RT-PCR

RNA was isolated from cells using TRIzol reagent. Two micrograms of RNA were reverse transcribed to cDNA with High- Capacity cDNA Reverse Transcription Kit. Quantitative real-time PCR was performed using iTaq Universal Probes Supermix and predesigned primers and probes (TaqMan® Gene Expression Assays). Relative mRNA expressions were calculated with the ΔΔCt method using HPRT or GAPDH as internal control.

3.12. Western Blot analysis

To evaluate protein expressions, cell lysates were run on 10% SDS PAGE. Western Blotting was performed with the use anti-Runx2, anti-ALP, anti-human ferritin heavy (FtH), light (FtL) chain antibodies, anti-aggrecan and anti-fatty acid binding protein 4 antibody, followed by HRP labeled anti rabbit IgG antibody. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system. After detection, the membranes were stripped and reprobed for GAPDH using anti-GAPDH antibody. Results were quantified by using Alpha DigiDoc RT quantification system.

3.13. Mice

All experiments were performed in compliance with institutional and national guidelines. Ten C57BL/6 mice (8–10 weeks old, male) were randomly divided into two groups. The iron-overload groupwas injected intraperitoneally with iron-dextran (200 mg/kg body weight, 3-times, every other day), while control mice were treated with PBS. Mice were sacrificed with CO_2 inhalation and perfused with 5 mL of ice-cold PBS, tibia and femur were harvested for analysis.

3.14. Isolation of compact bone osteoprogenitor cells (OPCs)

After harvesting and cleaning the tibia and femur, the epiphyses were removed. To release bone marrow, bones were fragmented and agitated in a mortar containing ice cold PBS supplemented with 2% FBS and 1 mmol/L EDTA. This washing step was repeated 5 times, and the bone marrow free bones were digested in 0.25% Collagenase Type I (5 min, RT). Then the bones were chopped into 1–2 mm pieces and digested for an additional 45 min at 37 °C. Finally the bone chips were washed, and the obtained suspension was filtered through a 70 μm cell strainer and centrifuged (300g, 10 min, RT).

3.15. 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. 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 h of death. 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 h at room temperature. Samples were centrifuged (2000 g, 5min, 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 s); supernatants were collected after a brief centrifugation (2000 g, 5 min, 4 °C).

3.16. Statistical analysis

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

4. Results

4.1. Pi and Ca induce mineralization of BMSCs in a dose-dependent manner.

First we tested whether elevated levels of Pi and Ca induce osteogenic differentiation of BMSCs. Media was supplemented with different concentrations of Pi (0–3 mmol/L) and Ca (0–1.2 mmol/L) and cells were cultured for 5 days. Alizarin Red staining revealed that while Pi and Ca alone did not induce ECM mineralization, when these triggers were applied together a dose-dependent mineralization of the ECM occurred, suggesting a synergistic effect of Pi and Ca in inducing ECM calcification. We observed a slight but significant increase in the Ca content of the ECM when the growth media was supplemented with 2 mmol/L Pi, and 0.6 mmol/L Ca. Higher concentrations of Ca and Pi induced a more robust increase in the Ca content of ECM. Importantly, these concentrations of Ca and Pi did not influence cell viability that was assessed by MTT assay. Based on these results, we used an osteogenic medium supplemented with 3 mmol/L Pi and 1.2mmol/L Ca to induce osteogenic differentiation of BMSCs throughout this work. Because the active, cell-mediated nature of ECM mineralization in VSMCs was questioned for decades, we wanted to be sure that Pi and Ca-induced mineralization is a cell-mediated process in BMSCs. To investigate this, we supplemented the osteogenic medium with the protein synthesis inhibitor, CHX. We found that CHX completely inhibited ECM mineralization, proving that de

novo protein synthesis is required for ECM mineralization and Ca deposition in BMSCs.

4.2. Iron inhibits Pi and Ca-induced mineralization of BMSCs in a dose dependent manner.

Previously it was shown that iron inhibits osteoblast activity as well as Pi-induced osteogenic trans-differentiation of VSMCs. To examine whether iron can inhibit Pi and Ca-induced osteogenic differentiation of BMSCs, we cultured the cells in osteogenic medium in the presence of excess iron (1–50 μmol/L), As revealed by Alizarin Red staining, iron caused a dose-dependent attenuation of mineralization, leading to complete inhibition at the concentration of 50 μmol/L. Next, we examined the effect of iron on Ca content of the ECM. Iron caused a dose-dependent decrease in the Ca content of the ECM. At the concentration of 50 μmol/L, iron lowered Ca content of the ECM of stimulated cells down to the level of nonstimulated cells. We examined the effect of iron on the level of Runx2. Osteogenic stimulation induced an about 1.6-fold increase in Runx2 mRNA level that was attenuated by iron. Importantly, iron at the concentration of 50 μmol/L decreased the level of Runx2 mRNA down to the level of unstimulated cells. Parallel with changes in mRNA levels, osteogenic stimulation increased the protein expression of Runx2 by 1.6-fold. Iron inhibited osteogenic stimuli induced upregulation of Runx2. Moreover, iron at the concentration of 50 μmol/L decreased Runx2 protein expression well below the Runx2 expression of control cells. Next, we determined the level of OCN, a bone-specific protein which expression is under the control of Runx2. Osteogenic stimulation robustly increased OCN level of the ECM, which increase was attenuated by iron, leading to complete inhibition at the concentration of 25 μmol/L.

4.3. Ferritin mimics the effect of iron in inhibiting ECM mineralization of BMSCs

Previously we found that iron inhibits VSMC osteogenic transdifferentiation and osteoblast activity, via the induction of ferritin. Our next question was whether the induction of ferritin is responsible for the anti-osteogenic effect of iron in BMSCs. As expected we found that iron induce a dose-dependent increase in both subunits of ferritin, FtH and FtL. Then we challenged BMSCs with osteogenic stimulation in the presence of ferritin at the concentrations of 1 and 2 mg/mL. Alizarin Red staining revealed that ferritin strongly inhibited ECM mineralization of BMSCs. This result was confirmed by measuring Ca content of the ECM.

Osteogenic stimuli induced an about 2-fold increase in Runx2 mRNA levels when compared to control cells. This response was completely abrogated in the presence of ferritin. Western blot analysis revealed that ferritin dose-dependently decreased the expression of Runx2. We found that OCN levels were very low in ferritin-treated samples. Comparison of ALP expressions in BMSCs under osteogenic conditions in the presence or absence of ferritin revealed that ALP expression of ferritin-treated cells was about ten times lower than in the absence of ferritin.

4.4. Iron overload downregulates the expression of Runx2 in osteoprogenitor cells (OPCs) in vivo

To investigate whether excess iron reduce osteogenic potential of OPCs in vivo, we induced iron-overload condition in C57BL/6 mice and measured mRNA level of the key osteogenic transcription factor, Runx2 and the iron storage protein FtH in compact bone-derived OPCs. As we expected iron overload caused a marked increase in FtH mRNA level, which was associated with a 60% decrease in Runx2 mRNA level in OPCs.

4.5. Osteogenic stimuli induce ECM mineralization of human lens epithelial cells (HuLECs)

To determinewhether osteogenic stimuli induce ECM mineralization in HuLECs, cells were cultured in calcification medium which was prepared by addition of Pi and Ca at the concentrations of 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. Pi alone induced ECM mineralization of HuLECs at the dose of 3.5 mmol/L, whereas Ca alone could not trigger ECM mineralization. Ca synergized Pi-induced ECM 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.

We exposed HuLECs to osteogenic medium (2.5 mmol/L Pi, 1.2mmol/L Ca) for 24 h 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. Western blot analysis confirmed the gene expression results and revealed that the osteogenic stimuli can trigger a 1.9-fold increase in Runx2 and a 5.5-fold elevation in Sox9 protein expressions.

Osteogenic treatment (7 days, 2.5mmol/L Pi and 1.2mmol/L Ca) resulted in a ~1.7-fold increase in ALP mRNA. This increase in ALP mRNA level was associated with increased protein expression of ALP.

OCN, being a major non-collagenous protein of fully mineralized bone-matrix. Therefore next we checked whether OCN gets also upregulated in HuLECs under osteogenic stimulation. We cultured HuLECs under control, and calcifying conditions for 7 days, and measured OCN mRNA levels. Osteogenic stimuli induced a 2-fold elevation in OCN mRNA levels. No OCN could be detected in the

EDTA-solubilized ECM of HuLECs cultured under basal conditions when Pi and Ca were applied together, a marked elevation in OCN levels was observed in the ECM of HuLECs.

4.6. 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 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. Expression of Slug mRNA increased in a time-dependent manner, reaching about 2.5-fold elevation on Day 5. We observed a slight decrease in Pax-6 mRNA level on Day 1 which was normalized by Day 5. We concluded that osteogenic differentiation shares some similarities with EMT.

4.7. Osteogenic differentiation markers 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. OCN was nondetectable 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.

5. Discussion

Our findings provide evidence that excess iron inhibits osteogenic differentiation and mineralization of BMSCs. To induce osteogenic differentiation of BMSCs here we applied Pi and Ca, the well-known and pathophysiologically relevant triggers of osteoblastic trans-differentiation of VSMCs. This kind of osteogenic

medium was used first time in the literature to induce osteogenic differentiation of BMSCs. The combination of high Pi and Ca induced faster osteogenic differentiation in BMSCs than the previously used osteogenic inductors (5 days versus 8-14 days). Iron caused a dose-dependent attenuation of mineralization, leading to complete inhibition at the concentration of 50 μmol/L. This result is consistent with the previous publication of Zarjou et al. in which it has been shown that the excess iron causes a total inhibition of osteogenic transdifferentiation of VSMCs at a concentration of 50 μmol/L. Previous study corroborates that iron decreases mineralization and demonstrates that this suppression is provided by iron-induced upregulation of ferritin.

We also showed that ferritin mimicked the inhibitory effect of iron in BMSC differentiation and ECM mineralization in agreement to that was found in osteoblasts. Additionally, in this work we investigated the *in vivo* effect of iron on osteogenic potential of OPCs. The crucial importance of Runx2 in osteogenic differentiation is highlighted by the finding, that Runx2 deficient mice completely lack differentiated osteoblasts, characterized by impaired bone formation, and die shortly after birth. We found that Runx2, the key osteogenic transcription factor is markedly downregulated in compact bone OPCs in mice with systemic iron overload. Our results provide a novel mechanism via which excess iron/ferritin could contribute to osteoporosis by impairing osteogenic differentiation of BMSCs and OPCs.

Several lines of evidence suggested an inverse relationship between osteogenic and adipogenic differentiation of MSCs Therefore we examined the role of iron in adipogenic differentiation of BMSCs expecting that iron may increase adipogenic potential of BMSCs. Interestingly, we found that iron does not influence adipogenesis, suggesting that the iron/ferritin system is an exception to this rule. Additionally, we described here that iron does not impair chondrogenic differentiation of BMSCs either.

Pathological osteo-/chondrogenic differentiation is involved in ectopic calcification, when precipitation and deposition of Ca-phosphate salts occurs in the form of HA in extraskeletal soft tissues. 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. Regarding eye pathology deposition of HA crystals in senile cataractous lenses has been shown by Raman microspectroscopy previously.

Vascular calcification is active process which can be studied in vitro. Elevated Pi and Ca are the most freqently used triggers to induce osteogenic differentiation and ECM mineralization of VSMCs in vitro. Therefore we used this well-establihed model, to investigate the process of osteogenic differentiation of lens epithelial cells in vitro. We showed that elevated Pi induces ECM mineralization of HuLECs, and Ca synergizes the effect of Pi. The osteogenic master transcription factor Runx2 has a critical role in osteogenic differentiation of VSMCs and recent results showed the upregulation of Sox9, the regulator of chondrogenesis in calcified vessels also occurs. Here we showed that HuLECs express both Runx2 and Sox9 under basal conditions. Moreover, osteogenic stimuli almost doubled the Runx2 and Sox9 expression in HuLECs compared to control cells.

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. In vitro studies revealed that ALP expression and activity are highly elevated in calcifying VSMCs. In agreement with this findong we observed that mineralization of HuLECs is accompanied by increased expression and enzymatic activity of ALP. Interestingly, ALP activity is significantly elevated in the plasma of cataract patients, though this phenomenon was linked to deteriorated liver function.

OCN is highly expressed in the fully mineralized bone-matrix. High OCN expressions have been found in mineralized extra-skeletal tissues such as calcified vessels and skin lesions in patients with calcific uremic arteriolopathy, cartilage and dental tissues. We revealed that osteogenic stimuli upregulate OCN expression in HuLECs as well. 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.

The main limitation of our work on BMSCs was that we did not investigate the signal transduction pathways in the background of the anti-osteogenic activity of iron / ferritin. We would like to carry out these experiments in the future. In connection with cataractogenesis, further work needs to be done to identify the clinically relevant inducers and inhibitors of lens calcification.

6. Conclusion

In this work we studied osteogenic differentiation of BMSCs and HuLECs. The aim of our work with BMSCs was to investigate the effect of iron overload on osteogenic differentiation of BMSCs, based on the evidence that suggest a link between chronic iron overload and bone abnormalities. Here we showed that excess iron inhibits osteogenic stimuli-induced osteogenic differentiation and ECM mineralization of BMSCs. Excess iron inhibited the osteogenic stimuli-mediated upregulation of Runx2, the master transcription factor of osteogenesis. Subsequently, iron inhibited upregulation of Runx2-regulated osteoblast-specific proteins in BMSCs. Iron overload in mice resulted in decreased Runx2 mRNA levels in compact bone-derived OPCs. Ont he other hand, we found no effect of iron on either adipogenic or chondrogenic differentiation of BMCSs.

The aim of our work with HuLECs was to investigate whether osteogenic stimuli induces osteogenic differentiation and ECM mineralization of HuLECs. Here we showed that osteogenic stimuli induces osteogenic differentiation and ECM

mineralization of HuLECs. We found that osteogenic stimuli increased the expressions of Runx2 and Sox9, the master regulators of osteogenesis and chondrogenesis respectively, and their target genes. Our work revealed that the genes activated during osteochondrogenic differentiation and EMT are partially overlap. OCN, an osteoblast-specific protein, was expressed in a few cataractous lenses, whereas no OCN could be detected in control lenses. Ca content was higher in human cataractous lenses, compared to non-cataractous controls and were highest in the OCN positive samples.

Overall our work can contribute to fuller understanding the mechanisms of iron-overload associated bone loss and lens calcification.

7. List of publications

DEBRECENI EGYETEM
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Jelölt: Balogh Enikő
Neptun kód: G2J99Q
Doktori Iskola: Laki Kálmán Doktori Iskola

A PhD értekezés alapjául szolgáló közlemények

1. **Balogh, E.**, Tolnai, E., Nagy, B. J., Nagy, B., Balla, G., Balla, J., Jeney, V.: Iron overload inhibits osteogenic commitment and differentiation of mesenchymal stem cells via the induction of ferritin.
   Biochim. Biophys. Acta-Mol. Basis Dis. 1862 (9), 1640-1649, 2016.
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   IF: 5.476
2. **Balogh, E.**, Tóth, A., Tolnai, E., Bodó, T., Bányai, E., Szabó, D. J., Petrovski, G., Jeney, V.: Osteogenic differentiation of human lens epithelial cells might contribute to lens calcification.
   Biochim. Biophys. Acta-Mol. Basis Dis. 1862, 1724-1731, 2016.
   DOI: http://dx.doi.org/10.1016/j.bbadis.2016.06.012
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További közlemények

3. Potor, L., Nagy, P., Méhes, G., Hendrik, Z., Jeney, V., Pethő, D., Vasas, A., Pálinkás, Z., **Balogh, E.**, Gyetvai, Á., Whiteman, M., Torregrossa, R., Wood, M. E., Olvasztó, S., Nagy, P., Balla, G., Balla, J.: Hydrogen Sulfide Abrogates Hemoglobin-Lipid Interaction In Atherosclerotic Lesion.
   Oxidative Med. Cell. Longev. 2018, 1-16, 2018.
   IF: 4.593 (2016)
4. Erdei, J., Tóth, A., **Balogh, E.**, Nyakundi, B. B., Bányai, E., Ryffel, B., Paragh, G., Cordero, M. D., Jeney, V.: Induction Of NLRP3 Inflammasome Activation By Heme In Human Endothelial Cells.
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   IF: 4.593 (2016)

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A DEENK a Jelölt által az iDEa Tudóstérbe feltöltött adatok bibliográfiai és tudománymetriai ellenőrzését a tudományos adatbázisok és a Journal Citation Reports Impact Factor lista alapján elvégezte.

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I recommend this work to my grandmother.