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The effect of low static magnetic field on osteogenic and adipogenic differentiation potential of human adipose stromal/stem cells

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ABSTRACT

The aim of this work was to investigate the effects of static magnetic field (SMF) on the osteogenic properties of human adipose derived mesenchymal stem cells (hASCs). In this study in seven days viability assay we examined the impact of SMF on cells proliferation rate, population doubling time, and ability to form single-cell derived colonies. We have also examined cells' morphology, ultrastructure and osteogenic properties on the protein as well as mRNA level. We established a complex approach, which enabled us to obtain information about SMF and hASCs potential in the context of differentiation into osteogenic and adipogenic lineages. We demonstrated that SMF enhances both viability and osteogenic properties of hASCs through higher proliferation factor and shorter population doubling time. We have also observed asymmetrically positioned nuclei and organelles after SMF exposition. With regards to osteogenic properties we observed increased levels of osteogenic markers i.e. osteopontin, osteocalcin and increased ability to form osteonodules with positive reaction to Alizarin Red dye. We have also shown that SMF besides enhancing osteogenic properties of hASCs, simultaneously decreases their ability to differentiate into adipogenic lineage. Our results clearly show a direct influence of SMF on the osteogenic potential of hASCs. These results provide key insights into the role of SMF on their cellular fate and properties.

1. Introduction

Regenerative medicine is currently one of the fastest developing fields of medical science which offers advanced solutions for treating a number of diseases. The potential application of stem cell-based therapies for repair and/or regeneration of various tissues will most probably become an alternative for currently applied medical solutions. The essential components of cell based therapies that stimulate the body's own repair system involve the application of autologous multipotent stem cells (MSCs) [1–3]. The most often applied multipotent stem cells, both as a research tool and in clinical trials, are those isolated from bone marrow multipotent stem cells (BMMSCs) and of adipose origin multipotent stem cells (ASCs). Although both of the above mentioned stem cell populations exhibit many similarities, ASCs are a potentially better candidate for regenerative medicine [2,4–6]. This is because of their high viability and ease of obtaining in large quantities with little donor site related complications or patient discomfort. Cellular therapies based on ASCs have been shown to be both safe and effective in pre- and clinical studies – both in veterinary, as well as human medicine [7–9] These stem cells posses the unique ability to differentiate into chondrocytes, osteoblasts and adipocytes, and recently also their differentiation potential into neuronal precursor cells has been demonstrated [10,11]. Beside self-renewal and multipotency ASCs have been demonstrated to display immunomodulatory effects by

Abbreviations: SMF, static magnetic field; hASCs, human adipose derived mesenchymal stem cells; MSCs, multipotent stem cells; BMMSCs, bone marrow multipotent stem cells; ASCs, adipose origin multipotent stem cells; MVs, microvesicles; BMP-2, bone morphogenetic protein 2; Col-I, collagen type I; OPN, osteopontin; OCL, osteocalcin; ALP, alkaline phosphatase; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CFU-E, colony forming unit efficiency; SEM, scanning electron microscope; PF, proliferation factor; PDT, population doubling time

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interacting both with natural, as well as adoptive immune responses [12,13]. Additionally, several studies indicate the importance of efficient intercellular signaling, which might be crucial in the context of successful tissue regeneration. Cells communicate with each other via secreted membrane derived microvesicles (MVs) [14–16]. As it has been shown, ASCs release MVs, which contain a wide range of molecules, including cytokines, growth factors, and microRNA (miRNA). They are involved in the improvement of donor cells response, proliferation and differentiation potential [17,18].

In todays' world, musculoskeletal disorders are one of the leading causes of disability [19], especially in the ageing populations. Bone defects can arise from trauma, congenital malformations or diseases such as arthritis or osteoporosis [20,21]. The results obtained by several research groups, including ours, have confirmed the positive therapeutic effect of ASCs in skeletal tissue repair in an animal model [7,8,22]. The positive clinical results of ASCs in the field of musculoskeletal system disorders might result from their ability to differentiate into osteoblasts that produce specific proteins and deposit minerals [23,24]. Thus, searching for factors able to enhance cell viability seems to be essential when application of MSCs in the field of regenerative medicine is considered. In our previous studies we have demonstrated that ASCs proliferation rate and their population doubling time (PDT) are distinctly increased when treated with a static magnetic field (SMF) [25]. It was previously shown that SMF might affect the behavior of cells [26]. It is assumed that SMF might effect biological free radicals [27] as well as influence the concentration of Ca^{2+} [28,29] and filaments. Thus, SMF might play an important role in intracellular signaling [27,28], as well as intercellular communication [29], as well as intercellular communication, and also possibly alter cellular function [30]. Besides improving cell proliferation, SMF has an analgesic and anti-inflammatory effect [31]. Beneficial effects and simplicity of application make SMF a complementary tool, which may support stem cell-based treatment. Effects of SMF as an osteoinductive factor which can support differentiation of stem progenitor cells into mature osteoblasts are still a poorly studied subject. Thus, we were interested if SMF might enhance the osteogenic differentiation potential of human adipose stem cells (hASCs).

The aim of the present study was to determine the activity of hASCs cultured under SMF conditions, including analysis of their proliferation rate, morphology, ultrastructure and osteogenic properties on protein as well as mRNA level. We established a complex approach, which enables obtaining information about SMF and ASCs potential in the context of differentiation into osteogenic and adipogenic lineages.

2. Materials and methods

All procedures were performed in accordance with manufacturers' instructions.

2.1. Static magnetic field exposure

The exposure system used in this study was previously described by Marędziak et al. [25]. A pair of permanent neodymium magnets with a known magnetic polarization vector was used to expose the cells to SMF. The intensity of the obtained SMF was measured using a Hall sensor and reached 0.5 T. Cell culture plates were placed in the gap between the magnets, and the exposure system was placed in a 37 °C CO_2 incubator.

2.2. Isolation of hASCs

Subcutaneous adipose tissue was collected from 10 female healthy donors that underwent total hip arthroplasty. Each donor signed an informed consent form prior to inclusion into the study. This study was approved by the Local Bioethics committee of the Wroclaw Medical School (registry number KB-177/2014), and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Adipose tissue samples were placed in sterile Hanks' balanced salt solution (HBSS, Sigma Aldrich Germany) and preserved at 4 °C after surgery, until further mechanical processing. Isolation of adipose-derived mesenchymal stromal cells was performed under aseptic conditions using well established methods [32,33]. In order to isolate the cells, tissue samples were incubated with collagenase type I (1 mg/ml; Sigma Aldrich, Germany). Cells were harvested by centrifugation and plated in a 25 cm^2 flask with Dulbecco's modified Eagle's medium (DMEM)/Ham' s F12 medium, supplemented with 10% fetal bovine serum (FBS) and with 1% penicillin/streptomycin/amphotericin b solution. Cultures were maintained at 37 °C in a humid atmosphere with 5% CO_2. The medium was changed every three days until cells reached approximately 80% confluence. Adherent cells were detached from the flask using TrypLE™ Express (Life Technologies, Poland). Cells were passage three times before the tests.

Table 1
Sequences of qPCR primers used for the amplification of human mRNA.

Gene name	Primer sequentions 5′–3′	Amplicon size (bp)	Accession number
Col-I	F: GTGATGCTGGTCCTGTTGGT R: CACCATCGTGAGCCTTCTCT	123	NM_000088.3
OPN	F: AAACGCCGACCAAGGTACAG R: ATGCCTAGGAGGCAAAAGCAA	213	U20758.1
BMP-2	F: ATGGATTCGTGGTGGAAGTG R: GTGGAGTTCAGATGATCAGC	349	KC294426.1
ALP	F: CGCGCTTGTGCCTGGA R: CCTGCTTTATCCCTGGAGCC	185	XM_006710546

2.3. Cell culture

The following conditions were investigated: (i) MF−: cells maintained without exposition to magnetic field (control conditions). (ii) MF+: ASCs cultured with exposition to magnetic field (both cultured in growth medium). (iii) Osteogenesis MF− cells cultured in osteogenic medium without exposition to magnetic field (control). (iv) Osteogenesis MF+: cells maintained in osteogenic medium, exposed to magnetic field. (v) Adipogenesis MF−: cells cultured in adipogenic medium, not exposed to magnetic field (control). (vi) Adipogenesis MF+: cells maintained in adipogenic medium under magnetic field.

All cells untreated with magnetic field were regarded as control for each condition.

For viability 7-days test cells were cultured in DMEM/Ham' s F12 medium, supplemented with 10% FBS and with 1% penicillin/streptomycin/amphotericin b solution. Cells from third passage were used for experimental purpose. For the test, cells were seeded into 24-well plates suspended at a concentration of 30×10^3 cells per well.

For osteogenic and adipogenic differentiation experiments, hASCs were cultured in osteogenic and adipogenic medium (STEMPRO®, Life Technologies, Poland). The adipo- and osteostimulation of cells was maintained in 24-well plates and the cells were inoculated at concentration of 30×10^3 cells per well. The media were changed every two days. Adipogenic and osteogenic stimulation was conducted for 14 and 21 days respectively.

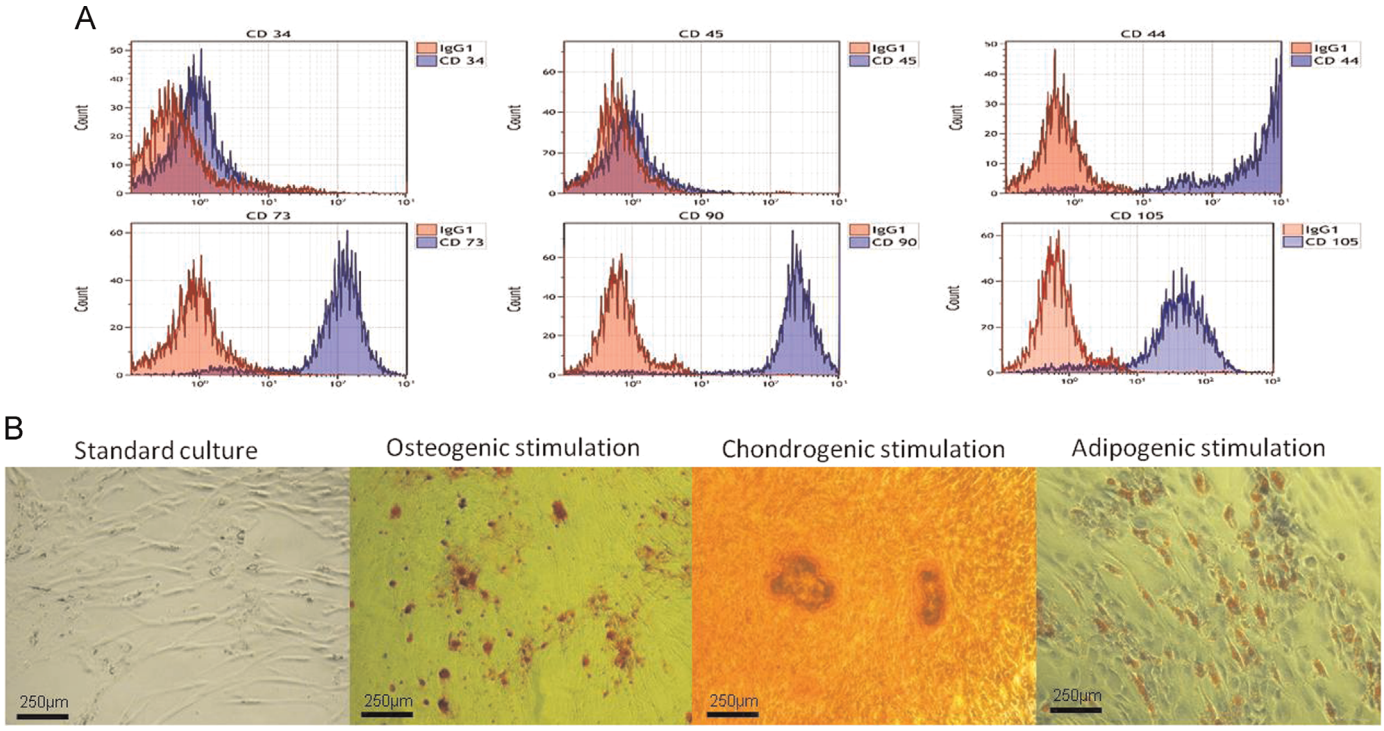

Fig. 1. (A) Representative flow cytometry results of human adipose derived mesenchymal stem cells (hASC) markers CD44, CD73b, CD90 and CD105, CD34, CD45. ASCs were positive for CD90, CD105, CD44, CD73b and negative for the leukocyte common antigen CD45 and hematopoeitic lineage marker CD34. The red area represents the expression of the IgG isotype control, and the blue lines depict marker expression. (B) Results of adipogenic, osteogenic and chondrogenic differentiation after 21 days of culture in specific induction media. Mineral calcium deposits visualized by Alizarin Red staining were clearly detected thereby indicative towards cells' ability to differentiate into osteogenic lineage. Representative photomicrographs of cultured hASCs stained with Oil Red O show lipid inclusions. Characteristic chondrocyte-like cells were observed and showed a strong orange signal after Safranin O staining. Undifferentiated cells did not exhibit any of these features. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Phenotyping and multilineage differentiation assay of hASCs

Prior to the experiment a total of 5×10^5 cells was analyzed by staining with CD45-APC, CD34-PerPC, CD105-FITC, CD90-PE, CD73b-PE, CD44-PE, CD29-PE (all antibodies purchased from BD Pharmingen). Fluorochrome-conjugated mouse immunoglobulin was used as isotype control. The stained cells were analyzed by Becton Dickinson FACS Calibur flow cytometer. At least 10000 cells were acquired. The samples were analyzed using FlowJo software (TreeStar).

In order to confirm the differentiation of hASCs into adipogenic, osteogenic, and chondrogenic lineages, cells from passage 3 were seeded in concentration 20×10^3 and cultured in tissue-specific media (STEMPRO® Life Technologies, Poland), according to the manufacturer's instructions. After 21 days of stimulation, specific stainings were performed-Oil Red O for adipogenesis quantification, Alizarin Red for osteogenesis and Safranin O for chondrogenesis. Stained samples were analyzed using an AxioObserver A1 inverted microscope (AxioObserverA1, Carl Zeiss, Jena, Germany), equipped with a Cannon PowerShot camera.

2.5. Cell viability and CFU-F

The number of viable cells was determined using the resazurin assay kit (TOX8, Sigma Aldrich). Viability of cells was estimated after 24, 48, and 120 h. All procedures, including calculation of proliferation factor (PF) and population doubling time (PDT), were performed according to a previously described method [33].

Colony-forming unit fibroblast (CFU-F) potential of single cells was determined by plating cells with a density of 1000 cells/well on a 6-well plate for 14 days and analyzed as previously described [18].

2.6. Examination of hASCs morphology and ultrastructure

Cell morphology, nuclei distribution and culture growth pattern were evaluated after 24, 48 and 120 h under an inverted, fluorescence microscope (AxioObserverA1, Zeiss) and a scanning electron microscope (SEM; EVO LS15, Zeiss). To visualize nuclei and actin filaments the cells were stained using DAPI and atto-488-labeled phalloidin respectively. For SEM, the cells were washed with distilled water and dehydrated in graded ethanol series. After 21 days the osteogenic differentiating Alizarin Red staining (for calcium deposits detection) was performed. Additionally, the presence of intracellular lipid vesicles, visible after 2 weeks of culture, was assessed by Oil Red O staining. For ultrastructure determination samples were sectioned into ultrathin slices (70 nm) and observed using an STEM detector at 10 kV filament tension. All procedures, both preceding and following microscopic observations, were previously described by our team [25,34].

2.7. Qualitative and quantitative analysis of cell matrix by SEM-EDX

Analyses of calcium and phosphorus concentration were performed using SEM combined with energy dispersive X-ray analysis (SEM/EDX). The quantax detector (Brüker) with 10 kV of filament tension was used to perform a line scan analysis of randomly selected cells. The obtained values were presented as weight percentage (wt%).

2.8. Quantitative bone morphogenetic protein 2, collagen type I, osteopontin, osteocalcin, and alkaline phosphatase, leptin adiponectin, insulin-like growth factor-II assays

The total concentration of bone morphogenetic protein 2 (BMP-2), collagen type I (Col-I), osteopontin (OPN) and osteocalcin (OCL)

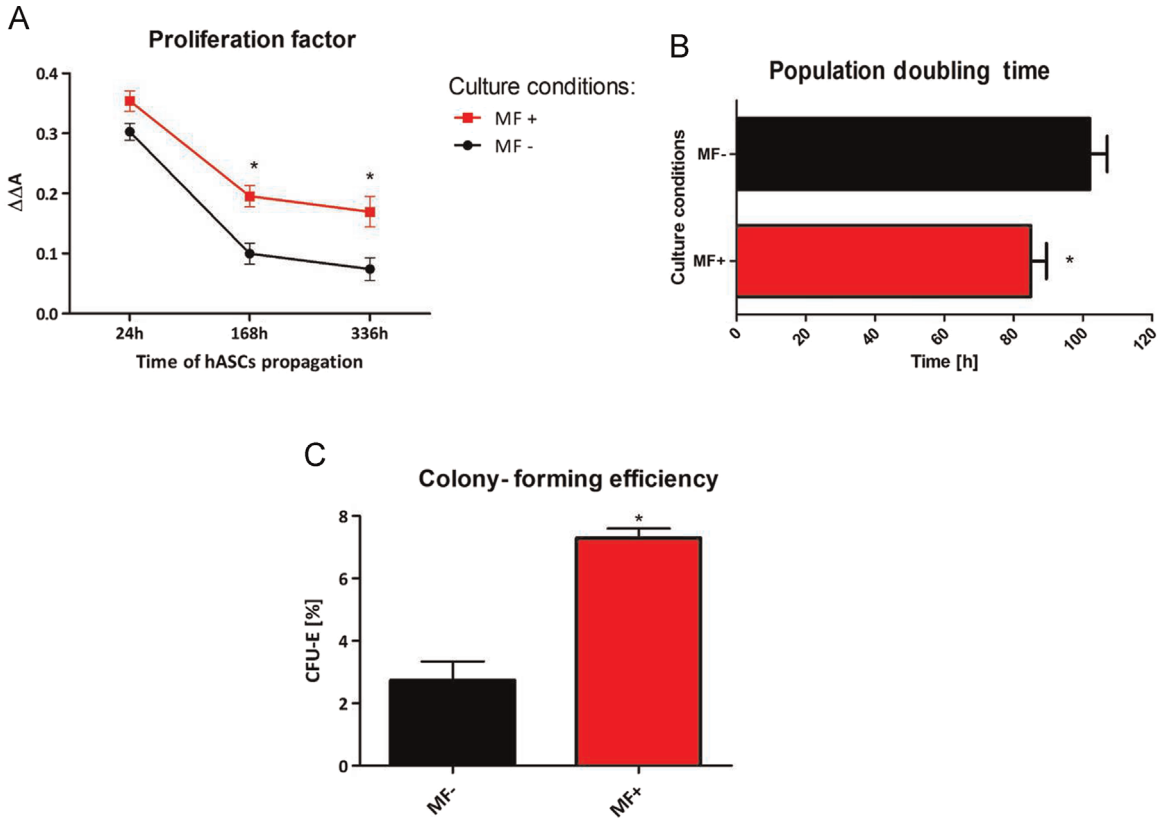

Fig. 2. Proliferation rate (A), population doubling time (B) and the colony forming efficiency (C) of human adipose derived MSCs cultures under SMF and in control conditions for 120 h. (A) Growth curves of hASCs show that cells cultured under SMF conditions were slightly higher than control cells at all measured time points. (B) Cells cultured under SMF reached population doubling time faster than those cultured in control conditions – 51.89 h vs. 45.15 h respectively. (C) Quantification of colonies after 14 days of culture, showed a significantly higher number of CFU-F in hASCs cultured in SMF. The asterisk marks statistical significance ($p < 0.05$).

was measured in collected supernatants derived from hASCs cultured in osteogenic medium under SMF and in control conditions. For this purpose ELISA assays were performed. The concentration of alkaline phosphatase (ALP) was quantified in supernatants using an ALP Colorimetric Assay Kit (Abcam, Cambridge, UK).

All ELISA kits were purchased from R&D Systems, except Col-I which was purchased from EIAab. The concentration of proteins was presented as a ratio of protein weight and supernatant volume (w/v).

2.9. Analysis of ALP, OPN, Col-I, and BMP-2 gene expression

Total RNA was extracted from hASCs' cultures using a phenol–chloroform method. DNA-free RNA was prepared using DNase I RNase-free kit (Thermo Scientific). 100 ng of total RNA was used for each reaction. Transcription of gDNA-free total RNA was reverse transcribed to cDNA using oligo(dT)15 primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Novazym). qRT-PCRs were carried out on a CFX Connect™ Real-Time PCR Detection System (BioRad) containing 5 µl of cDNA in a total volume of 20 µl, 500 nM primers and the SensiFast SYBR & Fluorescein Kit (Bioline). Analysis was performed using GAPDH invariant controls. Primer sequences are presented in Table 1.

2.10. Statistical analysis

Statistical analysis was performed using using GraphPad Prism 5 software. The unpaired *t*-test or a one-way ANOVA with Fisher's post-hoc test were used to test for statistical significance. Results are given as mean ± standard deviation (SD). A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Immunophenotyping and multipotency characterization of hASCs

Fig. 1A shows the immunophenotypic characterization of hASCs using flow cytometry. Cells expressed CD44, CD73b, CD90 and CD105 which are mesenchymal stem cells markers, but did not express the CD34 hematopoeitic lineage marker and the leukocyte common antigen CD45. Additionally stem cells isolated from human adipose tissue differentiated into adipogenic, osteogenic and chondrogenic lineages, which were verified by specific stainings (Fig. 1B).

3.2. Proliferation factor (PF), population doubling time (PDT) and colony forming efficiency (CFU-E) of hASCs during seven days culture under static magnetic field and in control conditions

Proliferation of hASCs was stable during a prolonged culture period as shown in Fig. 2A. At each time point, there was a difference between mean values of the two groups. Cells cultured under SMF had higher PF than cells cultured in control conditions. Differences were significant only after 168 h and 336 h ($p < 0.05$). Also cells cultured under SMF reached PDT faster (85.1 ± 4.5 h) than cells cultured in control conditions (102.2 ± 5 h) (Fig. 2B). CFU-E calculated for the control group (2.73 ± 0.61%) was significantly lower than that of hASCs cultured under SMF (7.3 ± 0.3%) (Fig. 2C).

3.3. Morphology and ultrastructure of hASCs during seven days culture under static magnetic field and in control conditions

Both hASCs cultured in control conditions (Fig. 3A–C) and under SMF (Fig. 3I–K) exhibited proper fibroblast-like, adherent and

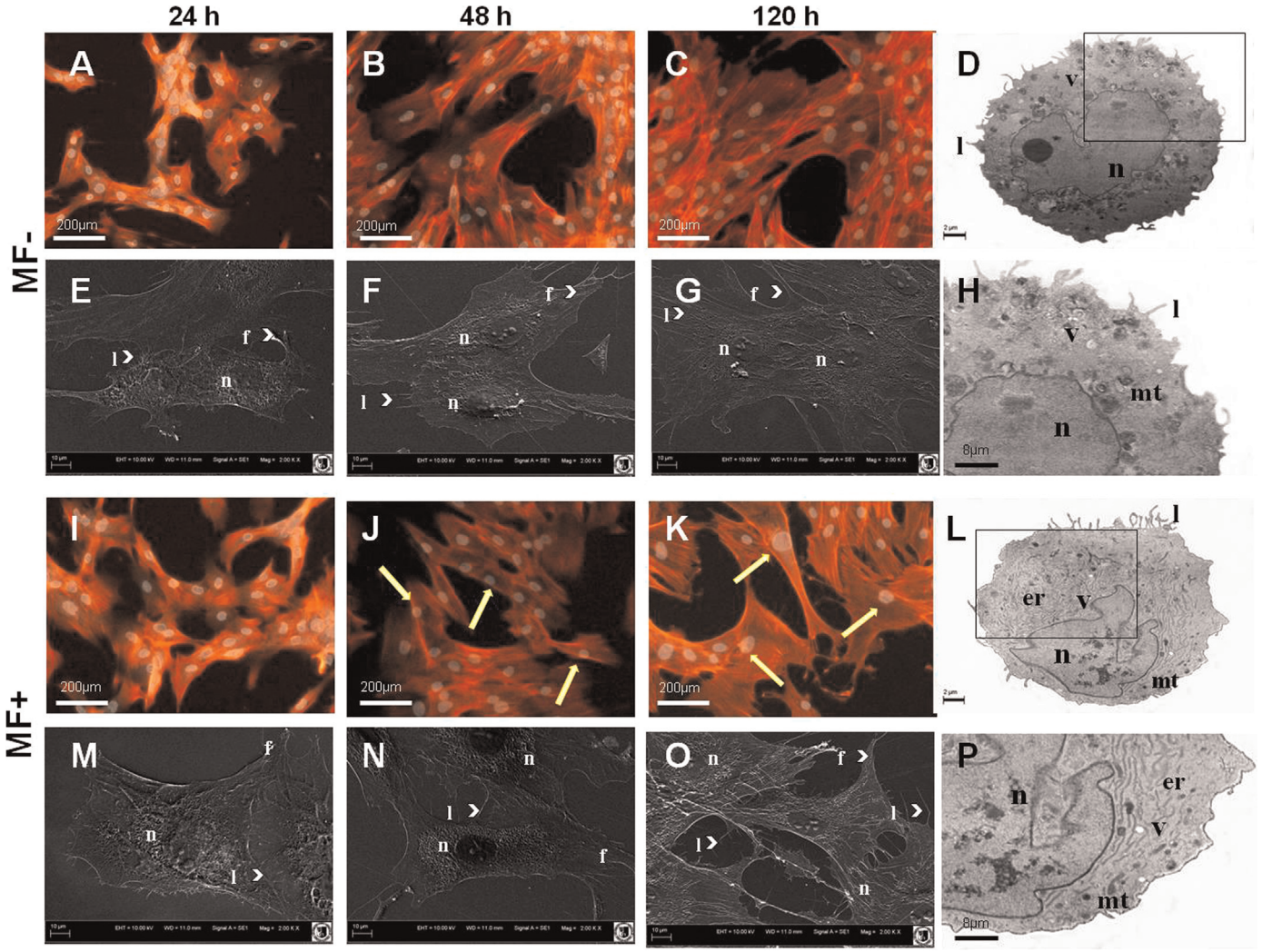

Fig. 3. Comparison of hASC morphology, nuclei distribution (A–C, F,G, I–K, M–O) and cell ultrastructure (D, H, L, P) under control conditions (A–G), and after exposure to SMF (I–P). Yellow arrows indicate displacement of the nuclei to the peripheral part of the cells. Cells exhibited proper fibroblast-like morphology. Cells cultured under SMF had a large number of filipodia and lamellipodia (M–O). TEM microphotographs (L, P) exhibited translocation of nuclei and several organelle. Appropriate scale bars are indicated on each panel. *n – nuclei; er – endoplasmic reticulum; mt – mitochondria; v – vacuoles; l – lamellipodia; f – fillipodia*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

elongated morphology. At the beginning of the experiment cells expressed similar growth patterns, which after 48 and 120 h differed significantly between experimental groups. DAPI staining indicated that cells cultured in the presence of SMF had nuclei located asymmetrically-towards one of the cell poles (Fig. 3J and K), whereas cells cultured in control conditions had their nuclei positioned centrally. This was clearly visible, especially after 120 h, and was confirmed by TEM observations. TEM microphotographs depict displacement of the nuclei to the peripheral part of each cell (Fig. 3L). Additionally, the cytoplasm of cells exposed to SMF contained several mitochondria and fewer rER stacks with dilated structure (Fig. 3L). SEM analysis showed that hASCs cultured under SMF were also characterized by formation of more Prod. Type: FTPthin cellular projections involved in exchange of cell information – filopodia and lamelopodia connecting neighboring cells (Fig. 3M–O).

3.4. Proliferation rate (PF) and population doubling time (PDT) of osteoblasts originated from osteoinduced hASCs

Growth curves of hASCs showed that cells cultured under SMF and in control conditions had similar proliferation rates which decreased during incubation time (Fig. 4 A). Cells cultured under SMF reached PDT faster than those cultured in control conditions – 97.5 h and 101.57 h respectively (Fig. 4B). Quantification of osteonodules formed after 21 days of cell culture under SMF, showed a significantly higher number per well than in samples cultured under control conditions (Fig. 4C).

3.5. Morphology and ultrastructure of osteoblasts originated form hASCs

In all investigated samples, under the osteogenic culture conditions, cells changed their appearance to rounder formed osteogenic nodules. In samples exposed to SMF (Fig. 5F and G) cellular clusters were denser, and cells came into closer contact with each other than in samples unexposed to SMF (Fig. 5A and B). Additionally, calcium hydroxyapatite crystals were present. The distribution of various shaped nanocrystals in the nodules cultured under SMF, exhibited a mosaic, regular pattern with numerous canals visible (Fig. 5H). SEM-EDX analysis also indicated that the mineralization ratio was appropriate in the SMF samples, and was about 2:1 in favor of calcium, whereas in the control samples an impaired balance was observed (Fig. 5K and L).

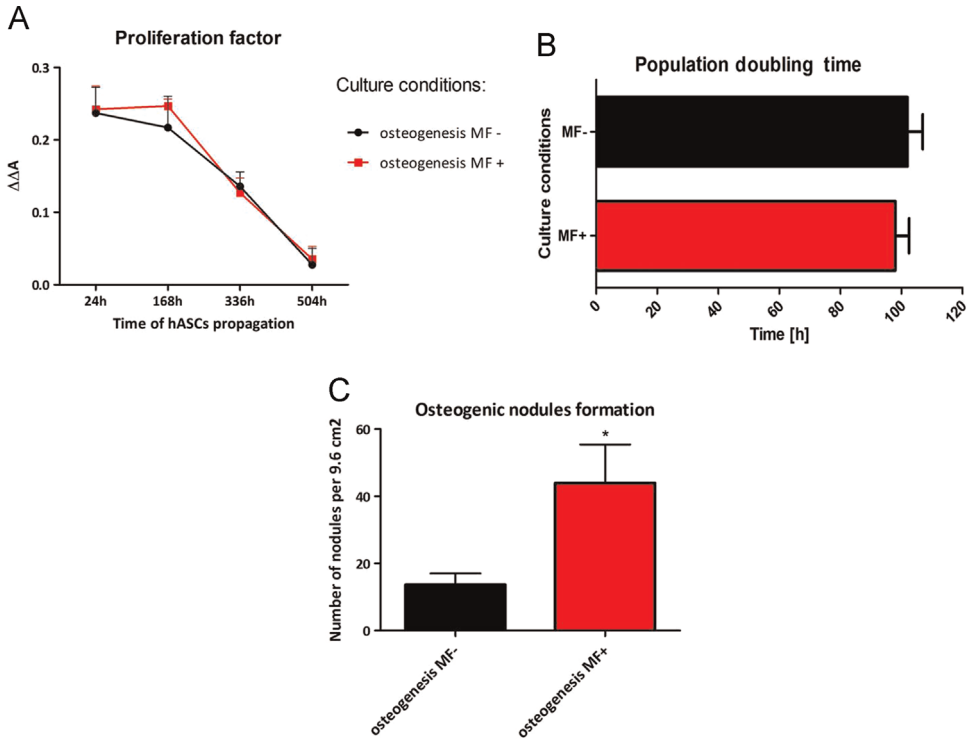

Fig. 4. Evaluation of proliferative activity (A) population doubling time (B) and quantification of the number of osteogenic nodules (C). (A) Growth curves of cells cultured in osteogenic conditions show similar proliferation rate which started to decrease after 168 h. (B) Cells exposed to SMF reached population doubling time faster than those cultured in control conditions. (C) Quantification of formed osteonodules after 21 days of culture under SMF, showed a significantly higher number than in samples cultured under control conditions. The asterisk marks statistical significance ($p < 0.05$).

Additionally TEM microphotographs have shown cells with typical osteoblasts morphology. After culturing under SMF cells had peripherally located, small nuclei and a smaller number of mitochondria (Fig. 5 I and J).

Positive results from Alizarin Red staining indicated that calcium deposits were accumulated in all investigated groups cultured in the osteogenic medium (Fig. 6A and B). Microscopy observations showed that the cellular aggregates in hASCs maintained under SMF demonstrated more intensive staining (Fig. 6B). In control cultures the positive reaction for Alizarin Red was noticeably less intense which was confirmed by quantifiable measurements of percent absorbed dye (Fig. 6C).

3.6. Osteogenic protein secretion and gene expression

The levels of released osteogenic proteins – Col-I, OPN, OCL, and BMP-2 were evaluated using ELISA. Fig. 7 shows the concentrations of all investigated proteins.

Interestingly, after colorimetric assay of ALP concentration, downregulation of ALP in cells cultured under SMF was observed (Fig. 7E). Relative mRNA expression levels of the early osteogenic markers – Col-1, BMP-2, and ALP, and the osteogenic late stage marker OPN were analyzed by RT-qPCR. The undifferentiated and unstimulated group served as the reference value. Alkaline phosphatase mRNA levels showed a clear increase after 14 days of SMF stimulation (Fig. 8D). Expression of BMP-2 and Col-1 was decreased, however without statistical significance (Fig. 8A and C). The expression of OPN increased significantly during osteoinduction.

3.7. Adipogenic differentiation of cells under static magnetic field

Microscope observations of adipocytes originated from hASCs cultured under SMF and in control conditions revealed that control conditions mostly enhance adipo differentiation. In the experimental group, Oil Red O staining exhibited small lipid deposits after SMF induction (Fig. 9B). After maintaining cells in adipogenic medium in control conditions, lipid droplets were highly abundant with large lipid vacuoles, positively stained by Oil Red O staining (Fig. 9A). These observations were consistent with the quantitative results obtained via Oil Red O absorbtion (Fig. 9C).

4. Discussion

The magnetic field is a common tool currently used with an increasing rate in medical practice and biotechnology [35–37]. Thus, there is a growing interest in understanding the effect of SMF on stem cells, especially in the context of their proliferative and differentiation potential. In this study we focused on the biological and functional characterization of hASCs and on the role of these cells in the context of osteogenic differentiation after culturing under the influence of SMF. The approach used to determine activity of hASCs cultured under SMF condition included analysing their morphology, proliferation rate, microvesicles' shedding and expression of genes related to bone formation, and revealed that SMF enhances hASCs activity.

In our previous study we have observed significant effects of 0.5 SMF on proliferative activity of canine and equine ASCs. The obtained results have shown that SMF influences the proliferation rate in a different manner depending on the cell population – it slightly reduces the proliferation rate of canine ASCs, but significantly enhances the proliferation activity of equine cells [25]. In this study, conducted using human cells, hASCs' PF was significantly higher in cells cultured under 0.5 SMF than in the control. We have also observed that the SMF field impairs the PDT of hASCs. In turn, Schäfer et al. [38] have shown that SMF (0.6 T) does not influence proliferation rate of human mesenchymal bone

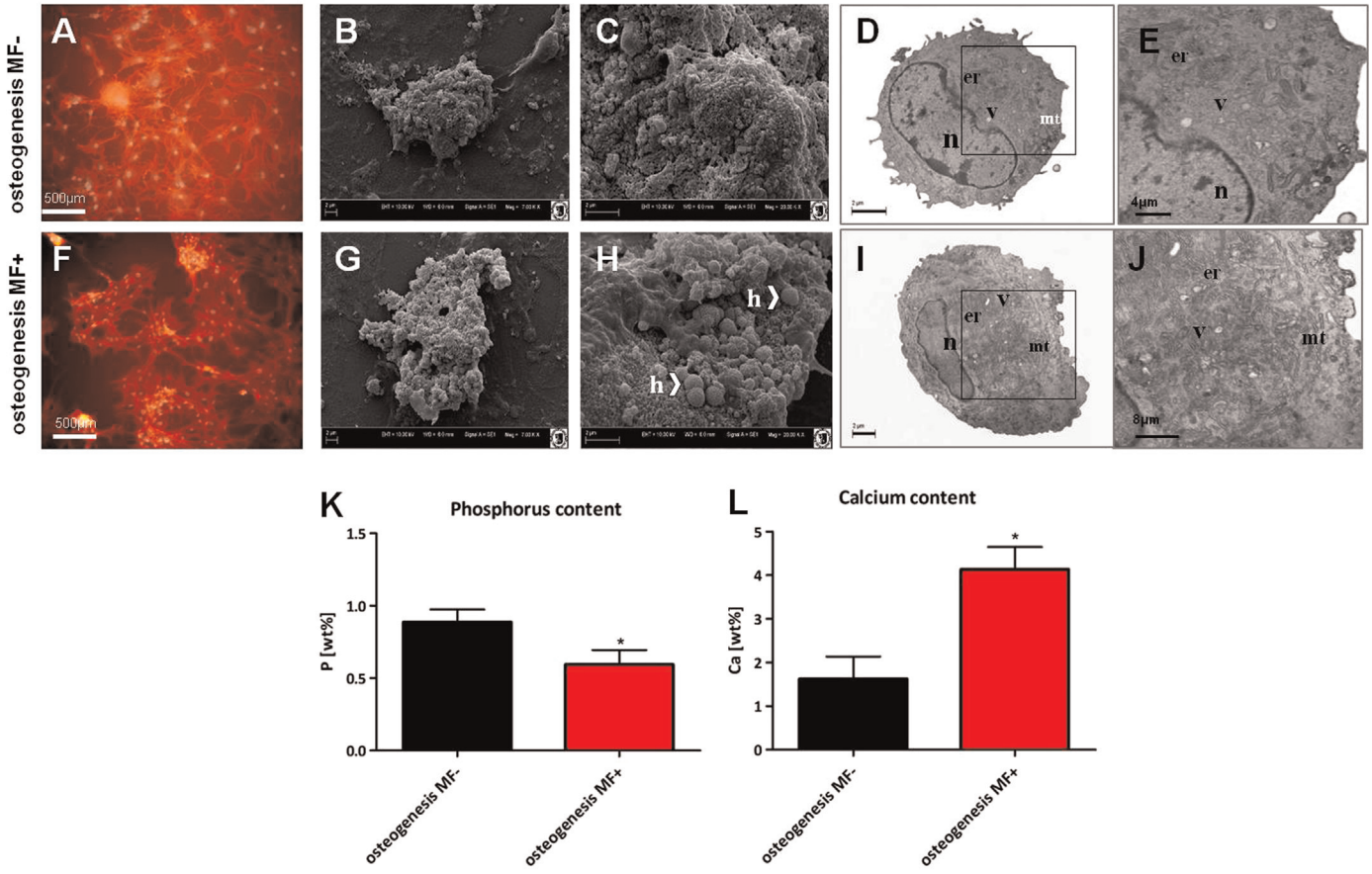

Fig. 5. Comparison of the morphology and ultrastructure of hASCs cultured in osteogenic conditions. Osteogenic differentiation shown by fluorescent staining (DAPI) in control (A) and SMF conditions (F) and SEM imaging ((B), (C) – control; (G), (H) – SMF exposed). SEM photographs clearly show the formation of hydroxyapatite nodules after SMF exposition (H) indicated by white arrowheads. Microphotographs (D) and (E) (control) and (I) and (J) (SMF exposed) compare the number and location of organelle. Appropriate scale bars are indicated in each panel. *n – nuclei; er – endoplasmic reticulum; mt – mitochondria; v – vacuoles; h – hydroxyapatites nodules.*

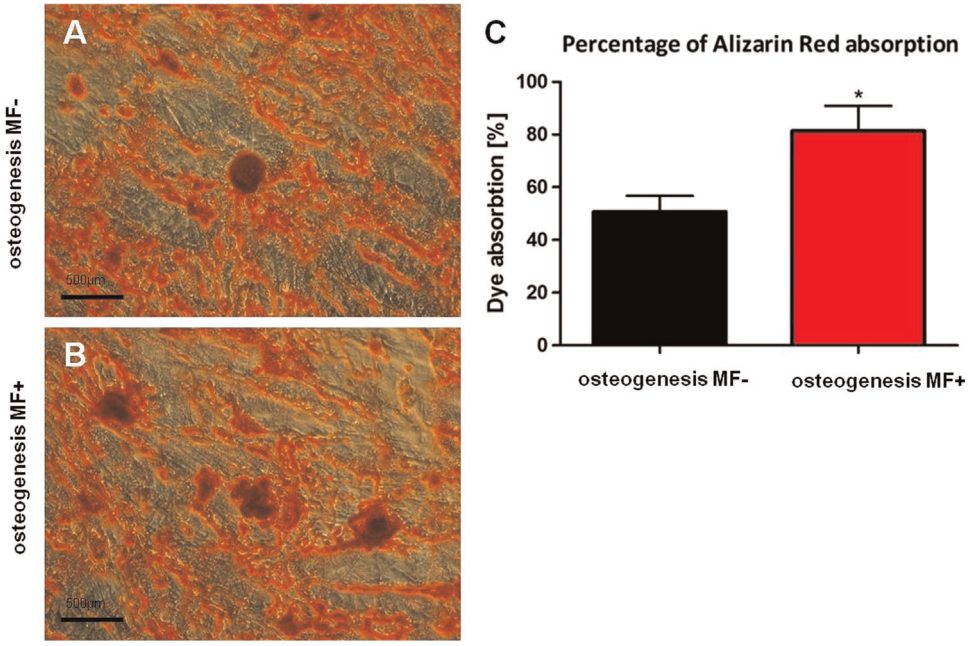

Fig. 6. Alizarin Red stained calcium deposits. Positive reaction for Alizarin Red is visible in both cultures – control (A) and SMF exposed (B). Quantitation of the staining confirmed by measurement absorbed dye (C).

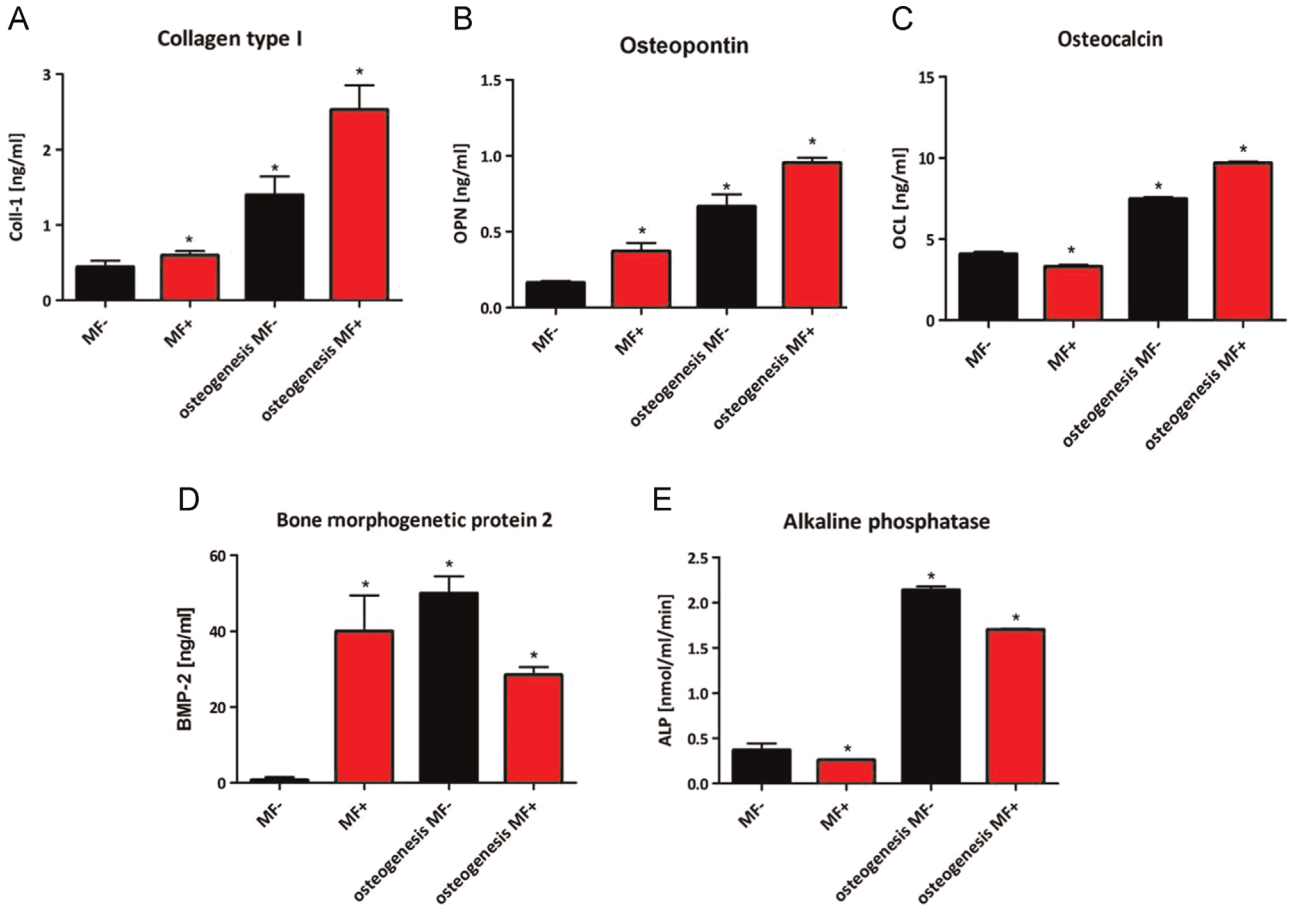

Fig. 7. Comparison of concentration of collagen type I (A), osteopontin (B), osteocalcin (C), bone morphogenetic protein 2 (D), and alkaline phosphatase (E) measured by ELISA. The asterisks mark statistical significance ($p < 0.05$).

marrow stem cells (hBMSCs). It seems that differences in the proliferative potential of stem progenitor cells cultivated under the influence of SMF might depend not only on the strength of the applied magnetic field but also on the stromal cells population type. It was also demonstrated that growth of human tumor cell lines (melanoma, ovarian carcinoma and lymphoma) cultured under extremely strong (7 T) SMF might be significantly inhibited [39]. Moreover, we did not observe differences in proliferative activity of hASCs cultured in osteogenic conditioned media under the influence of SMF in comparison to control culture. Our observations stand partially in agreement with the findings of Tsai et al. [40] who did not observe significant changes in proliferative activity of hBMSCs cultivated under the influence of a pulse electromagnetic field. Different cell types may respond differently to SMF due to discrepancies in their gene expression profiles. However, the underlying mechanisms of these effects and regulations of cell proliferation potential have been poorly investigated and not yet fully understood, and thus preclude from making definitive statements and comparisons.

In correlation with changes in the proliferation rate and PDT caused by SMF exposure, we have also observed an enhanced cell ability to form colonies when exposed to SMF. As Digirolamo et al. [41] have shown using hBMSCs, the number of colony-forming MSCs strongly correlates with their culture expansion ability and their differentiation potential. Moreover, Satomura et al. [42] showed that hBMSCs derived from a single colony differed from normal ones in many ways including cell morphology and colony structure [42].

In our study, besides the enhanced ability to form colonies, SMF had an effect on cell morphology. We have observed nuclei migration to one of the cell' poles and asymmetrical location of organelles. Other studies suggested that the effect of SMFs on cell morphology depends on cell type and field strength. Sato et al. [43] showed that culturing HeLa cells under 1.5 T SMF did not affect cell morphology, contrary to results obtained by Pacini et al. who reported significant changes in human skin fibroblasts cell shape, after exposure to 0.2 T SMF [44].

Changes in cell morphology in regards to cell shape and dimensions have drawn attention to the role of the biochemical response of cytoskeleton proteins to SMF exposure. As it was excellently described by Teodori et al. [30] SMF induces reorganization of microfilaments by regulating Ca^{2+} concentration, thus regulating cell orientation. The presence of Ca^{2+} can cause changes in the actin microdomain and thus lead to interaction with integrins and adhesion molecules responsible for cell shape and geometry. By modifying Ca^{2+} concentration and orientation of actin filaments, SMF can influence the polar distribution of cellular organelles [30,45]. We have observed this phenomenon in our study. Human ASCs cultured under SMF were characterized by organelles translocated to a specific pole. This led to the cell establishing new polarity. Moreover, cytoskeletal changes caused by SMF led to cell shape change and sequentially enhanced rounding of the cells. Electron microscopy observations demonstrated that cells exposed to SMF might form higher amounts of osteonodules with scattered matrix vesicles. The increased ability to form osteonodules under SMF could be correlated with higher colony forming efficiency. Besides a positive effect on cells' propagation, single-cell derived colonies have the greatest potential for differentiating into the osteogenic lineage. Cell cultures in single-cell colonies are phenotypically homogenous and contain cell

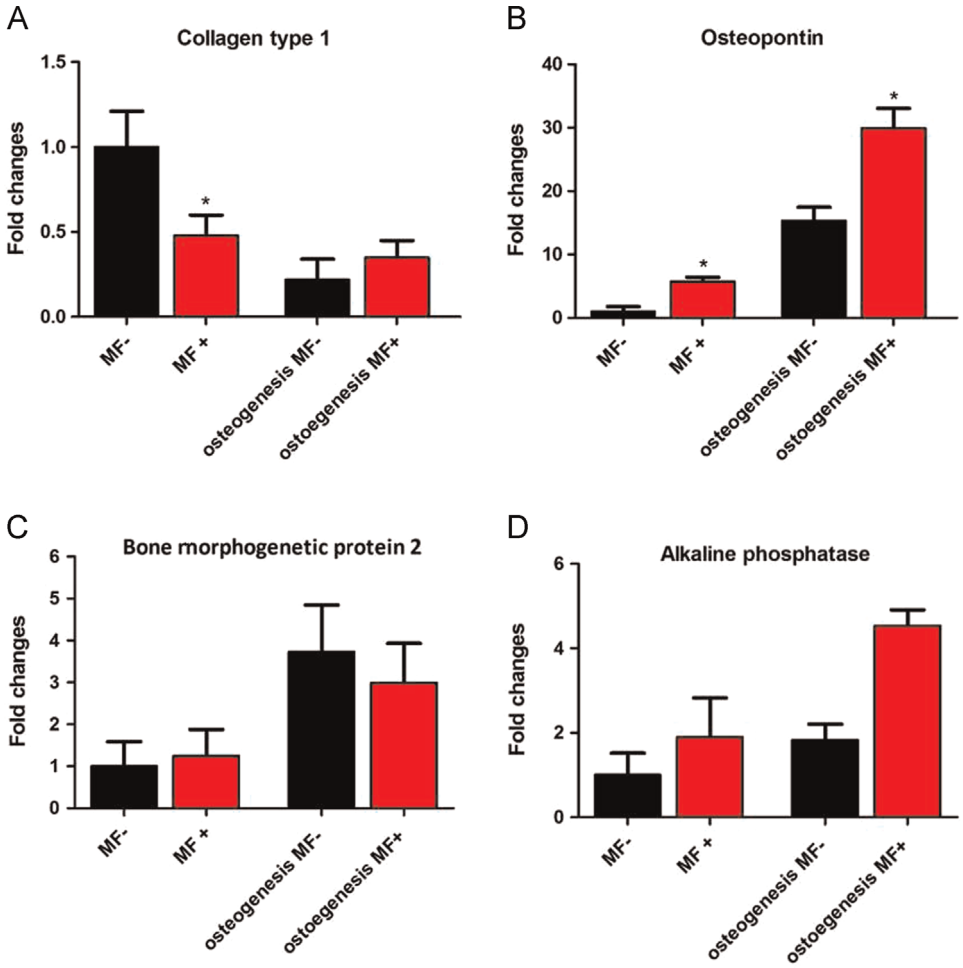

Fig. 8. Gene expression analysis comparing levels of collagen type I, osteopontin (B), bone morphogenetic protein 2 (C), and alkaline phosphatase (D). The asterisks mark statistical significance ($p < 0.05$).

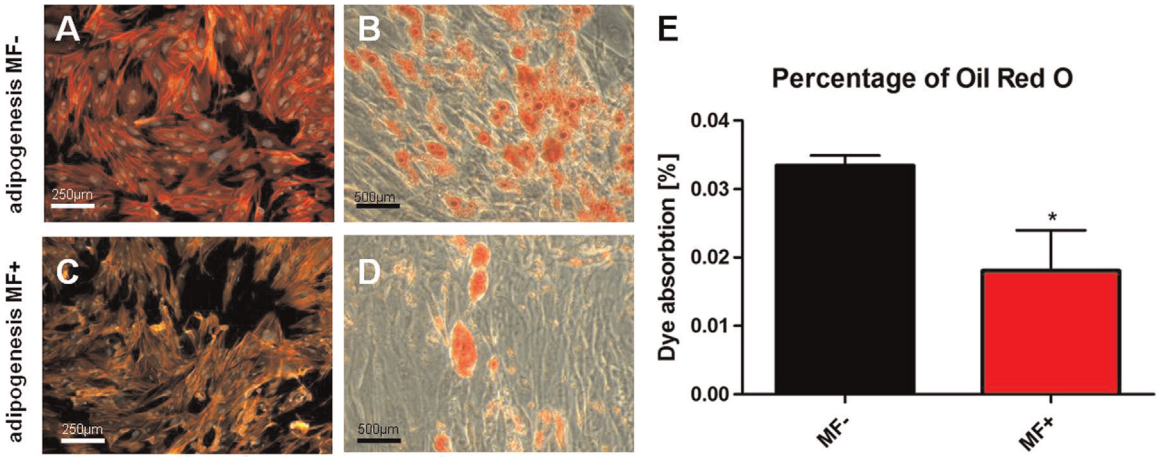

Fig. 9. Morphology of cells cultured with adipogenic medium ((A) – control conditions; (C) – SMF exposed) visualized by phalloidin and DAPI stainings. Representative photomicrographs show lipid droplets of cultured hASCs stained with Oil Red O ((B) – control, (D) – SMF-exposed) and percentage of Oil Red O staining absorption (E).

populations with identical differentiation potential [46,47]. Moreover, the ability to grow and expand in colonies also enhances cell–cell contact and may play a role in the expansion and differentiation of MSCs [48].

What is more, in this study, we have shown that osteonodules formed by cells cultured under SMF reached the mineralization ratio characteristic for osteoblastic cells. Since the extracellular matrix is a promoter of mineral deposition, hydroxyapatite formation indicates that cells exposed to SMF might progress to the development/maturation stage. In addition to bone nodule formation enhancement in cells exposed to SMF, SMF stimulates osteoblasts differentiation by regulating gene expression, as well as secretion of proteins specific for bone formation such as ALP, Col-I, OPN, OCL and BMP-2. In this study we have established that the osteogenic differentiation of hASCs was promoted by 0.5 T SMF. Moreover, we have observed that OPN and ALP gene

expression was upregulated under SMF conditions. Compared to control cells also gene expression of Col-I was upregulated under SMF. Furthermore, concentration of extracellular matrix proteins – Col-I, OPN and OCL was significantly higher in cells cultured under SMF in osteogenic conditions than in other study groups. In comparison, we have noticed lower concentration of ALP released from cells cultured under SMF exposition. The obtained data correlates with results obtained by other research groups. Huang et al. [49] reported an increased expression of Col-I in osteoblasts cultured under 0.4 T SMF whilst Feng et al. have shown increased ALP activity in cells cultured under a 0.4 T SMF. Although the effect of SMF exposition increases expression of genes responsible for osteogenesis (and this has been proven on osteoblasts cells) its effect on stem cells has not yet been fully determined [50,51].

In addition to stimulating osteoblast differentiation, as it was proven by Müller et al. [52] and as we have presented above, SMF might also influence changes in actin organization. Besides the influence on the osteogenic potential, a number of studies [53,54] have shown that actin cytoskeleton dynamics have been associated with the regulation of adipogenic differentiation. Therefore, we have also investigated the effects of SMF on differentiation into the adipogenic lineage. Our results have shown that exposition to SMF reduced the adipogenic differentiation of hASCs.

Mechanical and physical stress are transduced by integrins to the actin cytoskeleton to switch mechanical signals into biochemical pathways [53].

These findings provide evidence that SMF stimulation may play a modulating role in hASC osteogenesis. Our initial findings support the potential value of using SMF as a factor that might enhance the osteogenic potential of MSCs and simultaneously downregulate MSCs ability to differentiate into the adipogenic lineage. Our results indicates that SMF is involved in various mechanism associated with controlling signaling events. The static magnetic field as a non-toxic, osteogenic promoting agent is a promising candidate for regulating cellular functions of hASCs.

Competing interests

The author(s) declare that they have no competing interests.

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