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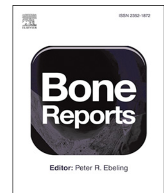
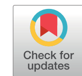


Effects of testosterone and 17β-estradiol on osteogenic and adipogenic differentiation capacity of human bone-derived mesenchymal stromal cells of postmenopausal women



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ABSTRACT

Progressive bone loss is a predominant symptom of aging and osteoporosis. Therefore, the effects of sex steroids (i.e. testosterone and 17β-estradiol) on the differentiation capacity of human bone-derived mesenchymal stromal cells (hMSCs), as progenitors of osteoblasts and adipocytes, are of particular interest. The objectives of the present study were, thus, to elucidate whether bone-derived hMSCs of postmenopausal women produce aromatase (CYP19A1) and, whether they modulate their differentiation behaviour in response to testosterone and 17β-estradiol (E2), in relation to their steroid receptor expression. Supplementation of testosterone resulted in a considerable formation of E2 under osteogenic and adipogenic culture conditions, whereas E2 synthesis remained minimal in the cells cultured in basal medium. Concomitant with high aromatase expression and 17β-estradiol formation of the cells cultured in osteogenic medium supplemented with testosterone, a distinct promotion of late-stage osteogenesis was found, as shown by significant matrix mineralization and a notable increase in osteogenic markers. These effects were abrogated by the aromatase inhibitor anastrozole. Under adipogenic conditions, testosterone reduced the occurrence of lipid droplets and led to a decrease in *PPARγ* and *AR* expression, independent of anastrozole. Regardless of the culture conditions, *ERα* was detectable whilst *ERβ* was not. In conclusion, aromatase activity is limited to differentiated hMSCs and the resulting 17β-estradiol enhances late osteogenic differentiation stages via *ERα*. Adipogenic differentiation, on the other hand, is reduced by both sex steroids: testosterone via *AR* and 17β-estradiol.

1. Introduction

Both, osteoblasts and adipocytes, derive from a common precursor, the mesenchymal stromal cell (MSC) (Duque, 2008; Kawai et al., 2012; Pino et al., 2012). MSCs reside in various tissues – including bone (Wagner et al., 2017; Wenisch et al., 2005, 2006) – and the major functional role of MSCs is to ensure cell turnover, repair and regeneration of tissues. They remain undifferentiated until differentiation into the respective resident cell types takes place (Kobolak et al., 2016). Differentiation of the MSCs towards the osteoblast or adipocyte lineage is a fine-tuned process controlled by a broad spectrum of factors predominating within the cells´ microenvironment (Hawkes and Mostoufi-Moab, 2018; Pino et al., 2012). The adipocytes of the bone marrow might play an important role in pathogenesis of osteoporosis (Duque, 2008). Osteoporosis is a multifactorial bone disease characterized by reduced bone quality and decreased mineral density (Khosla, 2010; Pino et al., 2012) due to an altered equilibrium of bone resorption and bone formation mediated by osteoclasts and osteoblasts. It has been suggested that an increase of adipocytes of the bone marrow and the concomitant reduction into osteoblast formation lead to decreased bone mass and, thus, to osteoporosis (Pino et al., 2012). Accordingly, MSCs from osteoporotic donors exhibited enhanced

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adipogenic differentiation capacity, whereas their proliferation capacity was reduced (Kawai et al., 2012). Moreover, osteogenic differentiation capacity of MSCs isolated from osteoporotic patients was retarded *in vitro* (Schaepe et al., 2017).

For bone loss observed in the course of osteoporosis, reduced systemic estrogen levels are considered a crucial factor (Duque, 2008) as estrogens play a pivotal role in bone homeostasis by inhibiting bone remodelling and suppressing bone resorption (Khosla, 2010; Ray et al., 2008; Syed and Khosla, 2005). Therefore, treatment with estrogens is well known to prevent bone loss (Bado et al., 2017). According to this, a 17β-estradiol-dependent increase of proliferation and expression of osteoblastic markers was seen in MSCs harvested from osteoporotic mice. Coincidentally, the rate of apoptosis was reduced (Zhou et al., 2001).

Estrogen is the sex steroid predominantly responsible for regulating bone metabolism in both women and men (Khosla and Monroe, 2018). Notably, estrogen can be produced locally in differentiated MSCs by aromatase (CYP19A1) (Kawai et al., 2012; Nelson and Bulun, 2001; Pino et al., 2006), and it has been proposed that the resulting local estrogen reservoir within the bone microenvironment might be sufficient in slowing the rate of postmenopausal bone loss in women (Nelson and Bulun, 2001).

Sex steroids (e.g. 17β-estradiol (E2) as well as testosterone (T)) mediate their effects on cells of the bone by activating estrogen (ERα and ERβ) and androgen receptors (AR), respectively. The receptors act as ligand-activated transcription factors (Jakob et al., 2010). However, the expression of ERs is tissue-dependent with a higher expression of ERα in cortical bone, while ERβ is preferentially expressed in trabecular bone (Bado et al., 2017). During osteogenic differentiation of rat calvarial cells *in vitro,* the ERβ is continuously expressed at low levels throughout the entire differentiation process, whereas ERα increases along with matrix maturation (Wiren et al., 2002). Therefore, ERα might play a particular role during the initial stages of bone stromal cell differentiation (Bado et al., 2017).

Although only a few studies have focused on the effects of sex steroids on osteoblast and adipocyte precursors up until now, it could be shown that 17β-estradiol stimulates osteogenic differentiation capacity of human bone marrow derived MSCs (Ray et al., 2008) – and even increases proliferation rates of MSCs harvested from osteoporotic mice (Zhou et al., 2001). Moreover, human MSCs exhibit increased osteogenic and adipogenic differentiation after direct exposure to 17β-estradiol (Hong et al., 2006).

The immediate precursor for aromatase-mediated 17β-estradiol synthesis is testosterone which is also crucial for bone metabolism (Ray et al., 2008). Testosterone reduces proliferation capacity of adipocytes (Ray et al., 2008), and inhibits adipogenic differentiation of the 3T3-L1 cell line (Singh et al., 2006). However, 5α-dihydrotestosterone (DHT), a considerably more potent agonist of the androgen receptor than testosterone (Jakob et al., 2010), is able to inhibit adipogenic differentiation of human MSCs (Gupta et al., 2008; Russell et al., 2018). In mouse bone marrow MSCs the effects of testosterone have been shown to be mediated by the androgen receptor (AR) (Russell et al., 2018) that is expressed in an age- and sex-independent manner in almost all tissues, including bone and bone marrow. Deletion of the AR in male mice (global-ARKO mice) results in increased fat mass whereas bone and muscle mass were decreased (Russell et al., 2018).

MSCs are considered the gold standard source of adult progenitor cells in the body (González-Vázquez et al., 2014). It is has been proven that sex steroids can induce distinct effects of MSCs. Therefore, the present study was performed to investigate the steroid receptor and aromatase expression of human bone-derived MSCs (Wenisch et al., 2005, 2006) of postmenopausal women who have not been diagnosed with osteoporosis. In addition, the researchers sought to explore the effects of testosterone and estrogen exposure on the osteogenic and adipogenic differentiation capacity of the cells. Details on the cell differentiation processes in response to sex steroids might help to understand cellular alterations that occur in the course of osteoporosis.

2. Materials and methods

2.1. Isolation and culture of human bone-derived mesenchymal stromal cells

Human bone-derived mesenchymal stromal cells (hMSCs) were isolated from spongiosa of femoral heads according to a previously published protocol (Wagner et al., 2017; Wenisch et al., 2005, 2006). The femoral heads of three female patients (mean age = 72.6 years, not osteoporotic) were provided from Agaplesion-Markus-Hospital (Department Orthopedics and Trauma Surgery, Frankfurt, Germany) after surgery due to coxarthrosis and the isolated cells were pooled. The study was approved by the local ethics committee of the Justus-Liebig-University Giessen (decisions 05/06 and 106/06).

The hMSCs of lower passages (passage 0–3) were cultured in standard cell culture medium (α-MEM (minimum essential medium) (Life Technologies – Thermo Fisher Scientific, Darmstadt, Germany) containing 20% FBS (Biochrome, Berlin, Germany) and 100 U/ml penicillin/streptomycin (PAA Laboratories, Pasching, Austria) in a humidified atmosphere. For the experiments, cells of the fourth passage were used.

The cells were seeded in 24 well plates (1.9 cm^2 per well) with a density of 20 000 cells per well and cultured in basal medium (BM) containing α-MEM (Life Technologies – Thermo Fisher Scientific, Darmstadt, Germany), 10% FBS (Biochrome, Berlin, Germany) and 1% P/S (PAA Laboratories, Pasching, Austria).

After 24 h the cells were transferred to osteogenic (OM) and adipogenic medium (AM).

The OM consisted of BM supplemented with 10 $mmol{\cdot}L^{-1}$ β-glycerophosphate, 0.06 $mmol{\cdot}L^{-1}$ ascorbic acid, and 0.1 $\mu mol\ L^{-1}$ dexamethasone (all Sigma-Aldrich, Steinheim, Germany).

The AM was prepared by supplementation of BM with 1 $\mu mol\ L^{-1}$ dexamethasone, 0.2 $mmol{\cdot}L^{-1}$ indomethacin, 0.5 $mmol{\cdot}L^{-1}$ IBMX (3-isobuthyl-1-methylxanthine), and 5$\mu g{\cdot}mL^{-1}$ ITS Liquid Media Supplement (all by Sigma-Aldrich, Steinheim, Germany).

In order to analyze aromatase activity of the cells, all the three media (BM, OM, AM) were supplemented with 0.1 μM testosterone (4-Androsten-17β-ol-3-on, Steraloids, Inc., Wilton, USA) dissolved in ethanol (see Table 1) – hereafter referred to as BMT, OMT, AMT. Additionally, the BMT, OMT and AMT were supplemented with the aromatase (CYP19A1) inhibitor anastrozole (A) (1μM, catalog no. S1188, Selleckchem, Muenchen, Germany) dissolved in DMSO – referred to as BMTA, OMTA and AMTA. The sufficient anastrozole concentration presently used to inhibit aromatase activity has been defined in preliminary experiments by using concentrations of 0.1 μM, 1 μM, and 10 μM anastrozole (data not shown). Cells in all media were observed and photographed on day 1 and day 21 of culturing with Leica DMIL and corresponding software LAS (Leica Microsystems, Wetzlar, Germany)

Table 1
List of the media used for culturing of the MSCs.

Medium / Abbreviation	Medium supplementation and final concentration
BM	BM
BMT	BM + 0.1 μM testosterone
BMTA	BM + 0.1 μM testosterone + 1 μM anastrozole
OM	OM
OMT	OM + 0.1 μM testosterone
OMTA	OM + 0.1 μM testosterone + 1 μM anastrozole
AM	AM
AMT	AM + 0.1 μM testosterone
AMTA	AM + 0.1 μM testosterone + 1 μM anastrozole

Basalmedium (BM), Osteogenic medium (OM), Adipogenic medium (AM), testosterone (T), anastrozole (A).

for the documentation of cell morphology.

2.2. Determination of 17β-estradiol concentrations in cell culture media

For the measurement of 17β-estradiol (E2) concentrations of the cell culture media, pooled samples were taken on day 1, 7, 14, and 21 of the culture period. Medium changes were done 24 h before the medium collection.

Radioimmunological determination of E2 concentrations was performed as previously described (Hoffmann et al., 1992; Klein et al., 2003) after extraction of the samples with toluene. The antiserum applied was directed against 17β-estradiol-6-carboximethyloxim–BSA. Cross-reactions of 1.3% were observed for estrone, 0.7% for estriol and below 0.01% for the non-phenolic steroids tested including testosterone. A minimum detectable concentration of 7.3 pmol/l was documented. Intra- and inter-assay coefficient of variability (CV) were found to be 7.1% and 17.6% respectively. Final E2 concentrations were calculated after subtraction of the blank measured in unconditioned medium.

2.3. Detection of aromatase (CYP19A1) by immunofluorescence

Cells were seeded on glass plates and fixed with 4% PFA in PBS at day 1 and 21. After permeabilisation with 0.25% Triton X (Carl Roth GmbH, Karlsruhe, Germany) in TBS and blocking with BSA they were incubated with a murine monoclonal antibody against human aromatase (CYP19A1) (catalog no. SM2222PS, lot no. A150260BH, Clone H4, Acris antibodies, Herford, Germany) 1:100 in background reducer (DAKO, Hamburg, Germany) over night. Following several washing steps, blocking was performed with goat and human serum in BSA. After incubation with the Alexa 488 conjugated secondary antibody (DCS, Hamburg, Germany, goat anti mouse) the nuclei were stained with Hoechst Dye (H33258, Sigma-Aldrich, Steinheim, Germany).

As a positive control for the staining MCF-7 cells (kindly provided by Prof. S. Mazurek, Institute of Veterinary Physiology and Biochemistry, Justus-Liebig-University of Giessen, Giessen, Germany) was used (data not shown). Negative controls were performed without the primary antibody.

Pictures were taken with the Axio Observer Z1 (40x objective in oil) and the corresponding software AxioVision Release 7.4.8 (Carl Zeiss MicroImaging GmbH, Goettingen, Germany).

2.4. Visualization of matrix mineralization by Alizarin S staining

Matrix mineralization (i.e. detection of Ca^{2+}) in the course of osteogenic differentiation was made visible and quantified by *Alizarin S* staining of the cells. Briefly, the cells were fixed with 4% paraformaldehyde (PFA) in PBS after 21 days in culture and washed three times with double distilled water. Afterwards, the staining procedure was performed by means of 1% *Alizarin S* solution (Carl Roth GmbH, Karlsruhe, Germany) and pictures were taken (Leica DMIL and Leica MC 170HD, Leica Microsystems, Wetzlar, Germany). Subsequently the *Alizarin S* was removed with *cetylpyridinium chloride* (CPC, Sigma-Aldrich, Steinheim, Germany). The optical density of the solution was measured in a plate reader (Sunrise™, Tecan) at $\lambda = 562$ nm (reference wavelength $\lambda = 630$ nm) to define the amount of *Alizarin S*.

This result was determined in relation to the number of cells per well. For this aim the CPC was dissolved and the cells were incubated with *sulforhodamin B* (SRB, Sigma-Aldrich, Steinheim, Germany). After several washing steps with 1% acetic acid the SRB was solved in unbuffered tris (hydroxymethyl) aminomethane (TRIS)-solution (Sigma-Aldrich, Steinheim, Germany). The optical density of this solution was measured at $\lambda = 565$ nm (reference wavelength $\lambda = 690$ nm) with the plate reader (Sunrise™, Tecan).

The relative extracellular matrix production was calculated as the ratio of the optical density of *cetylpyridinium chloride* divided by the optical density of *TRIS*-solution.

The measurements were taken as quadruplicates.

2.5. Detection of ALP activity

The activity of the alkaline phosphatase (ALP) after osteogenic differentiation with OM, OMT, and OMTA was measured in cell lysates after 21 days in culture. After lysis the cells were incubated with 2 mg/ml 4-Nitrophenolphosphat in ALP-activity buffer. After 60 min the reaction was stopped with NaOH and the optical density was measured, with the plate reader (Sunrise™, Tecan), at $\lambda = 405$ nm (reference wavelength $\lambda = 620$ nm). The measurements were repeated three times in three independent wells per medium.

2.6. Detection of lipid droplets by oil red O staining

In order to make lipid droplets visible within the cells' cytoplasm after incubation of MSCs in adipogenic differentiation media, *Oil Red O* staining was used. After 21 days of differentiation the cells incubated on glass plates (duplicates) were fixed with 4% PFA in PBS and washed three times. Then they were incubated with *Oil Red O* staining solution and counterstained with hematoxylin. Micrographs were taken using the light microscope Axiophot (Carl Zeiss MicroImaging GmbH, Goettingen,Germany) provided with a digital camera (Leica microsystems, Ltd type DFC320, Bensheim, Germany). 5 overviews per glass plate were taken with 2.5 fold magnification for cell counting of *Oil Red O* positive cells.

2.7. Measurement of gene expression by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Gene expression of differentiation markers was analyzed by means RT-qPCR. Sample collection for RNA isolation was done on day 1 and 21 of cultivation. For extraction the innuPREP RNA Mini Kit (Analytic Jena AG, Jena, Germany) was used according to the manufacturer´s protocol. The extracted RNA was stored in liquid nitrogen.

Preparation of cDNA was performed according to a previously published protocol (Glenske et al., 2014). The RT-qPCR was performed by the use of the QiagenQuanti Fast SYBR Green PCR Kit (Qiagen, Hilden, Germany) combined with the QuantiTect Primer Assays (Qiagen, Hilden, Germany) as described previously (Glenske et al., 2014). The following QuantiTect Primer Assays were used: androgen receptor (*AR*, Qiagen-ID:QT00073451), bone morphogenetic protein 2 (*BMP2*, Qiagen-ID: QT00012544), connexin 43 (*Cx43*, Qiagen-ID: QT00012684), estrogen receptor 1 (*ERα*, Qiagen-ID: QT00044492), estrogen receptor 2 (*ERβ*, Qiagen-ID: QT00060641), osteocalcin (*OCN*, Qiagen-ID: QT00232771), peroxisome proliferator-activated receptor gamma (*PPAR*γ, Qiagen-ID: QT00029841), runt-related transcription factor 2 (*Runx2*, Qiagen-ID: QT00020517). Glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*, Qiagen-ID: QT00079247) was used as the housekeeping gene.

Data were analyzed by using the ΔΔCt-method. The gene expression of cells cultivated in media with T as well as T with A (BMT, BMTA, OMT, OMTA, AMT, AMTA) were calculated in correlation to the corresponding media without supplements (BM, OM, AM).

2.8. Statistics

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

Measured E2 concentrations and gene expression were analysed by a two-way ANOVA for the variables time and medium. In case of error probability < 0.05 matching media were compared applying the Mann-Whitney-Test. Statistical evaluation of the data from the semi-quantification of Alizarin S staining, ALP activity, and counting of Oil Red O positive cells per medium were calculated with a one-way

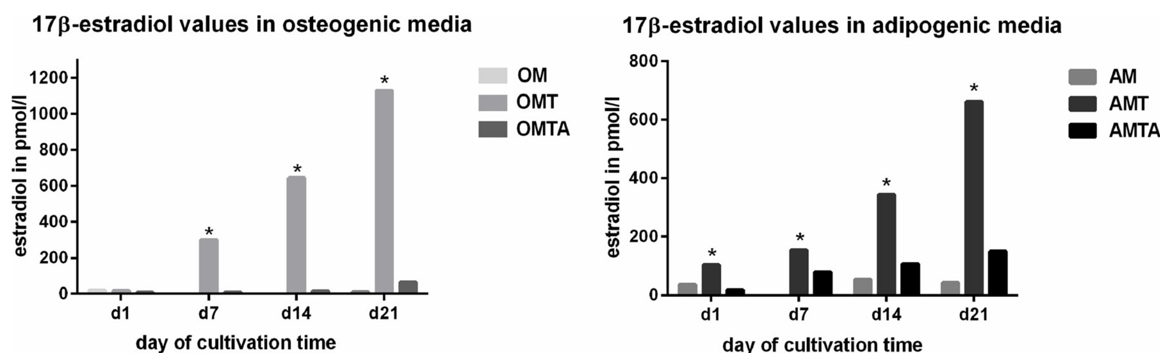

Fig. 1. 17β-estradiol concentrations measured by means of radioimmunological assays in pooled samples of osteogenic and adipogenic media (OM and AM) during the cultivation of hMSCs. The estradiol concentrations of the testosterone containing media OMT and AMT increased significantly over time. (* $p < 0.05$). Supplementation of the OMT and AMT with anastrozole (e.g. OMTA, AMTA) inhibited the formation of 17β-estradiol.

ANOVA across the corresponding media.

3. Results

3.1. Concentrations of 17β-estradiol in the cell culture media

In the course of cultivating the cells within the three basal media (BM, BMT, BMTA), the concentrations of 17β-estradiol (E2) slightly increased from < 7.3 pmol/l (minimum detectable concentration) on day 1, to 47.3 pmol/l and 47.0 pmol/l on day 21, in the BMT and BMTA respectively (data not shown). In the osteogenic differentiation media the initial E2 concentration on day 1 was 21.6 pmol/l in the OM, and 7.7 pmol/l in the OMTA (Fig. 1). In these cultures no increase of the E2 concentrations could be observed over the time. However, within the OMT the concentrations increased constantly and significantly ($p = 0.0159$) over the time – up to 1129 pmol/l at day 21. Therefore, it can be concluded that cells of OMT were able to aromatize testosterone and to produce 17β-estradiol, whereas testosterone was available for the cells in the OMTA but 17β-estradiol was not.

No considerable increase of E2 concentration was observed when MSCs were incubated in AM or AMTA, whereas a significant increase over the time ($p = 0.0317$) could be found in the AMT – approximately up to 660.9 pmol/l on day 21.

3.2. Morphology of hMSCs in response to testosterone and 17β-estradiol

After 24 h in differentiation media without supplements (w/o), with testosterone, and with testosterone and anastrozole, no differences of the cell morphology between all culture conditions are detectable. All cells revealed the typical spindle-shaped morphology (Fig. 2). Even after 21 days in all basal media (BM, BMT, BMTA) spindle-shaped cells were observed irrespective of the supplements. During cultivation in osteogenic media hMSCs developed into cuboid cells in all conditions and visible extracellular matrix in OM (w/o) and OMT (with testosterone). Cells cultivated under adipogenic conditions with and without supplements showed a typically flattened morphology. Nevertheless, in AM (w/o) a lot of lipid droplets occurred, whereas in the supplemented media (AMT and AMTA) they are difficult to find (Fig. 2).

3.3. Aromatase expression by immunofluorescence

By means of immunofluorescence, specific aromatase signals were exclusively observed in the cytoplasm. Irrespective of the composition of media, no aromatase staining could be detected on day 1. However, on day 21 cells cultivated in OMT and AMT exhibited clear immunostaining (Fig. 3). Signals in MSCs after incubation in BM, OM, AM and BMT, BMTA, OMTA, and AMTA were of minimal intensity or absent.

3.4. Matrix mineralization

Alizarin staining of Ca^{2+} within the mineralized matrix at the late stage of osteogenesis revealed an expected staining in the osteogenic medium OM. A stronger and more intensive staining occurred in the osteogenic medium supplemented with testosterone (OMT) whereas in OMTA (with testosterone and anastrozole) the staining is weaker (Fig. 4).

Semiquantification of alizarin via CPC revealed a highly significant increase of calcium deposition of the cultures incubated OMT compared to cultures of the OM, OMTA and all basal media (in all cases $p < 0.001$). Furthermore, a highly significant decrease of alizarin staining occurred in OMTA compared to OM ($p < 0.001$) (Fig. 5).

3.5. ALP activity

ALP activity occurred in early stages of the osteoblast differentiation process. The detection of ALP activity in osteogenic differentiated cells showed a slight increase in cells cultivated in OMT (with testosterone) in comparison to cells cultivated in OM without reaching significant levels (optical density of 1.13 versus 1.07). Furthermore, a highly significant decrease in cells cultivated in OMTA (with testosterone and anastrozole) compared to OM and OMT was detectable (Fig. 6).

3.6. Lipid droplet formation

Adipogenic differentiation was made visible by means of Oil Red O staining of the cells incubated in the AM, AMT, and AMTA on day 21 (Fig. 7). Adipocytes could be observed in all cultures. However, a significantly higher number of cells containing lipid droplets was observed in cultures of the AM compared to AMT and AMTA. Between the testosterone supplemented media AMT and AMTA, no difference in Oil Red O positive cells was detectable (Fig. 8).

3.7. Gene expression

By means of RT-qPCR in hMSCs cultivated in the basal media (BM, BMT, BMTA) expression of the following genes *Runx2, BMP2, OCN, Cx43, PPARγ, AR*, and *ERα* could be detected on basal levels. The *ERβ* was not detectable. Moreover, the gene expression of cells cultivated in BMT and BMTA showed no differences, thus, indicating that gene expression of the cells cultivated in the different basal media was not affected by the supplements testosterone and anastrozole in itself.

Gene expression of the cells on day 1 and day 21 after osteogenic differentiation in combination with testosterone (OMT) and with testosterone and anastrozole (OMTA) was normalized to gene expression detectable in the cells of the osteogenic medium (OM) (Fig. 9 A).

Significant increase of expression up to day 21 of the osteogenic markers *Runx2, BMP2*, and *Cx43* could be seen (Fig. 9 A) when the cells

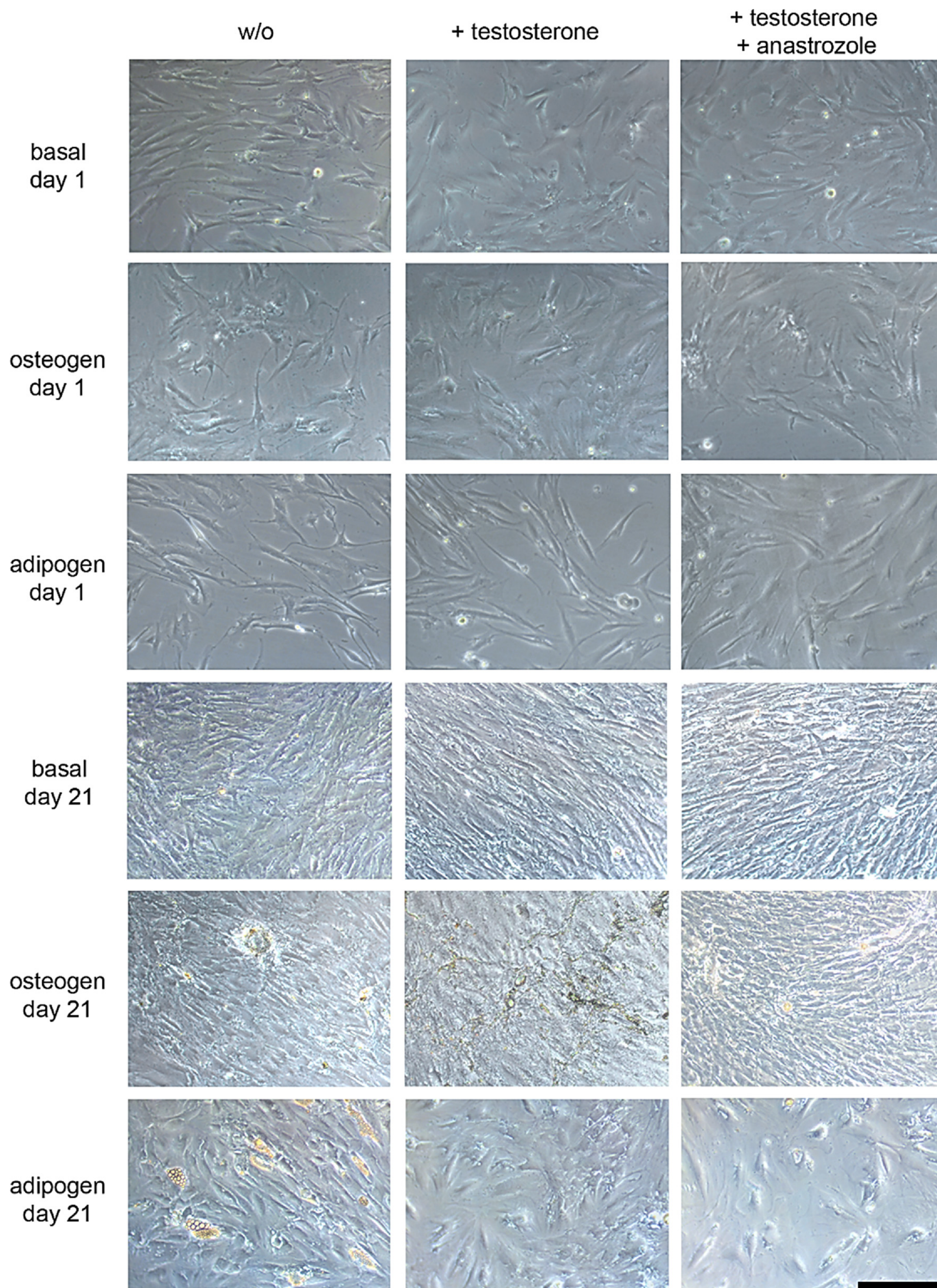

Fig. 2. Representative micrographs of native hMSCs cultivated in basal, osteogenic, and adipogenic media without testosterone (w/o), supplemented with testosterone and with a combination of testosterone and anastrozole after 1 day and 21 days cultivation period. Scale bar = 200 μm applies to all micrographs.

were cultivated in the OMT – the medium containing high concentration of 17β-estradiol (Fig. 1). According to this, cells of the OMT revealed a higher expression of *OCN* on day 21 compared to cells within OMTA. Furthermore, a significantly higher *Cx43* expression was observed on day 21 in comparison to day 1, and in contrast to the cells cultivated in the OMTA, further indicating enhanced osteogenic differentiation of cells incubated within the OMT.

The expressions of the *AR* and the *ERα* showed a slight tendency to increase over the time in the OMT (x-fold expressions day 1 vs. day 21: *AR* 1.0 vs. 1.15; *ERα* 0.81 vs. 0.94) and the expression was slightly

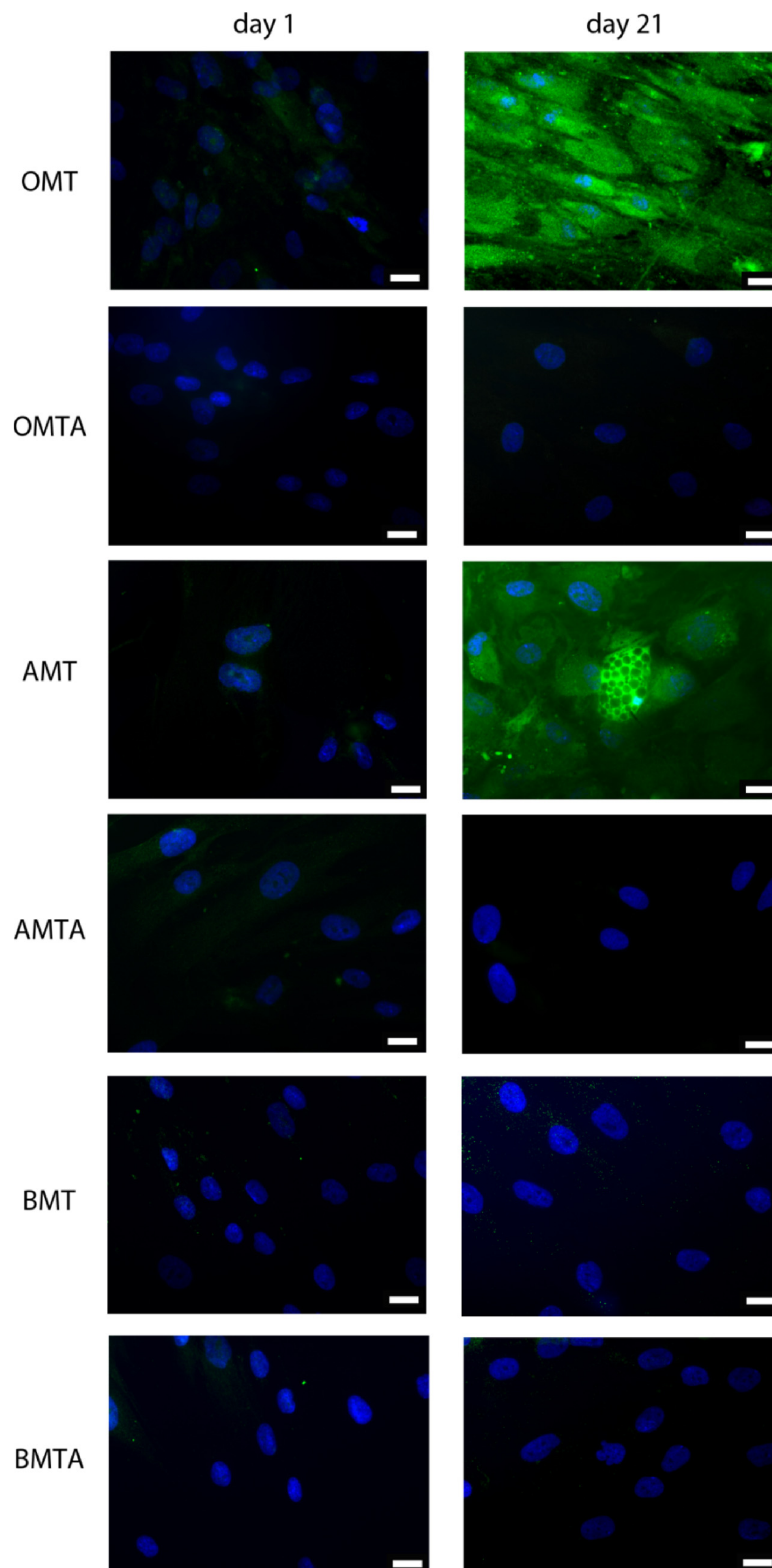

Fig. 3. Exemplary micrographs of the detection of aromatase (CYP19A1) by immunofluorescence (green staining) in hMSCs on day 1 and 21. The MSCs were cultivated in the osteogenic (OMT, OMTA), the adipogenic (AMT, AMTA), and basal media (BMT, BMTA) (for media composition see Table 1). For every medium and day the immunofluorescence was performed in duplicates. Nuclei are stained in blue. Scale bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

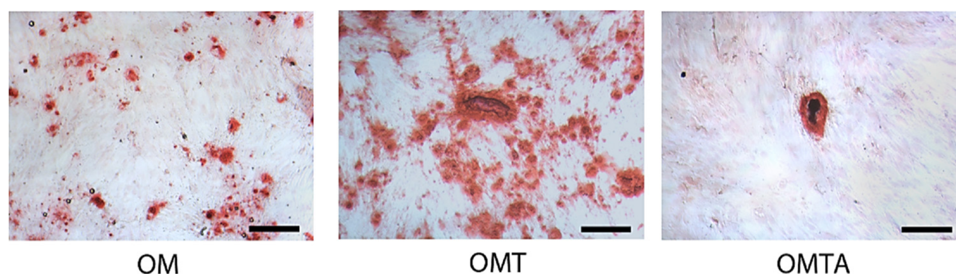

Fig. 4. Alizarin staining of the cells cultivated within the different osteogenic media. Staining for every medium was done in triplicates and from these results typical micrographs were shown exemplarily. Only in cultures of the OMT a significant increase of calcium deposition was detectable. Matrix mineralization was distinctly reduced when aromatase activity was blocked by means of anastrozole (OMTA). Scale bar = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

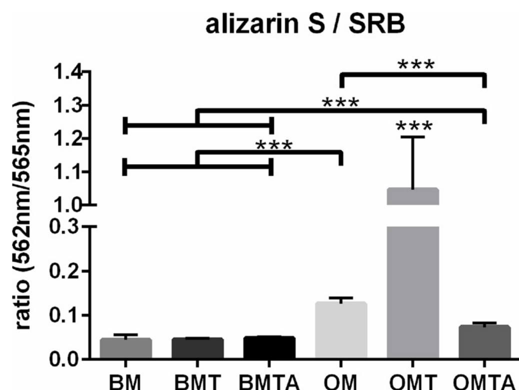

Fig. 5. Semiquantification of alizarin staining in relation to relative cell number measured with SRB in all basal (BM, BMT, BMTA) and osteogenic media (OM, OMT, OMTA). All measurements were done in quadruplicates and measurements were repeated three times. Data is presented as mean ± SD. *** $p < 0.001$.

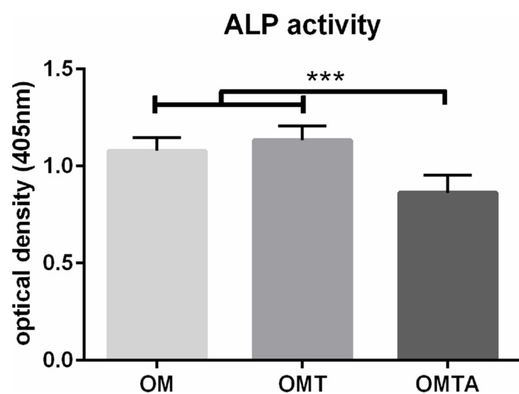

Fig. 6. Quantification of ALP activity in cell lysates of hMSCs cultivated in OM, OMT, and OMTA for 21 days. Cells cultivated in OMTA revealed a highly significant decrease of ALP activity. All measurements were done in triplicates and measurements were repeated three times. Data is presented as mean ± SD. *** $p < 0.001$.

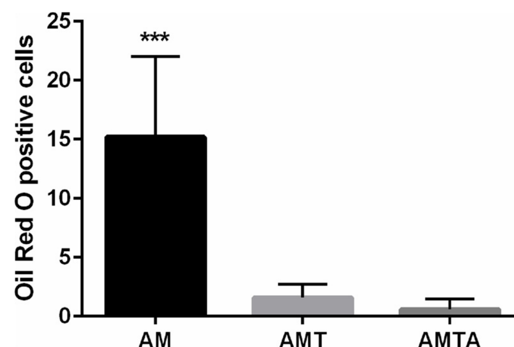

Fig. 8. Counting of Oil Red O positive cells in the three adipogenic media AM, AMT, and AMTA. 5 overviews per glass plate were taken with 2.5 fold magnification for cell counting of Oil Red O positive cells. Data is presented as mean SD. *** $p < 0.001$.

higher in OMTA than in the OMT. However, the expression of the *ERα* decreased in the course of time in cells of the OMTA (x-fold expression day 1 vs. day 21: 1.32 vs. 0.93, not significant), whereas the expression of the AR remained unchanged over the time in the OMTA.

Gene expression of the cells incubated within the adipogenic medium supplemented with testosterone (AMT) as well as with testosterone and anastrozole (AMTA) was calculated in comparison to gene expression of the cells incubated in adipogenic medium (AM) (Fig. 9 B).

A highly significant decrease of *PPARγ* could be observed up to day 21 when the cells were cultured in the AMT and the AMTA (Fig. 9 B), indicating suppression of PPARγ-mediated adipogenic differentiation in response to testosterone with high concentration of 17β-estradiol (AMT, compare Fig. 1), and testosterone alone, in case of the AMTA.

AR-gene expression decreased significantly up to day 21 in cells of both the AMT and the AMTA. *ERα*-gene expression of the cells of the AMT and AMTA was only detectable on day 1.

Taken together, the results demonstrate that an increase of the osteogenic markers could be observed when the cells were cultured in combination with testosterone with high 17β-estradiol levels – such as in the case of the OMT.

Downregulation of the adipogenic marker *PPARγ* occurred in response to high levels of 17β-estradiol with testosterone (AMT) and testosterone (AMTA) – suggesting that adipogenesis might be

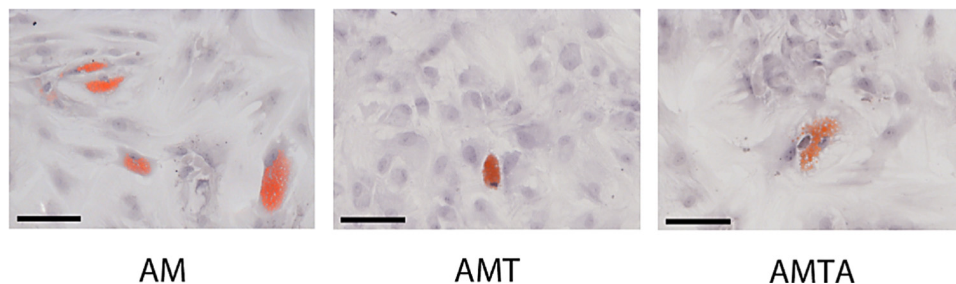

Fig. 7. Oil Red O staining of the cells cultivated in different adipogenic media (AM, AMT, AMTA). Staining for every medium was done in triplicates and from these results typical micrographs are shown exemplarily. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

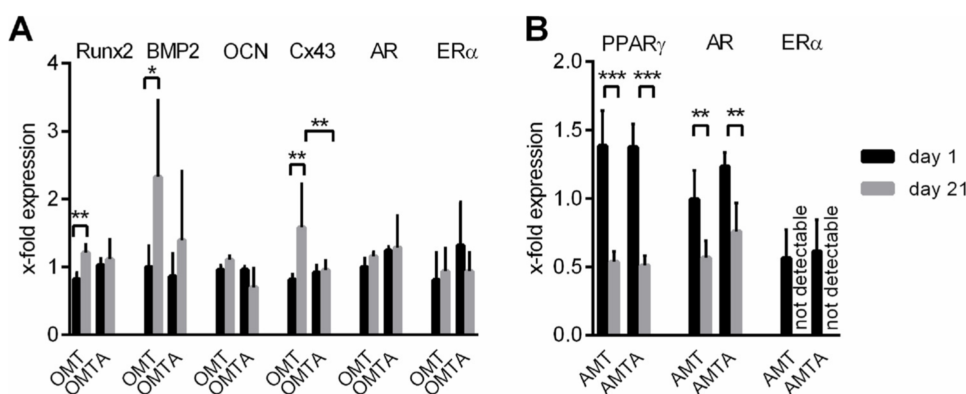

Fig. 9. Gene expression on day 1 and on day 21 of hMSCs A) incubated in osteogenic differentiation media supplemented with testosterone (OMT) or with testosterone in combination with anastrozole (OMTA). B) hMSCs incubated within the adipogenic differentiation media supplemented with testosterone (AMT) and with testosterone and anastrozole (AMTA). Gene expression was detected from three parallel wells per medium in triplicates and repeated at least once. The results presented are based on relative gene expression levels measured by RT-qPCR (ΔΔCt-method) and are calculated as x-fold expression in comparison to the corresponding differentiation medium without further supplements (OM and AM, respectively). Data is presented as mean ± SD. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

suppressed by both hormones.

4. Discussion

The main results presented in this study have shown, that 17β-estradiol promotes the late stages of osteogenesis in human bone-derived MSCs of postmenopausal women without osteoporosis while both sex steroids, testosterone as wells as 17β-estradiol inhibited their adipogenesis.

The effects of sex steroids on cell behaviour of MSCs have been investigated in a few studies (Gupta et al., 2008; Hong et al., 2006, 2009; Leskelä et al., 2006; Zhou et al., 2001), however, most analyses have been performed by using standard culture conditions and/or direct supplementation of 17β-estradiol, testosterone, or DHT. In this present study adipogenic and osteogenic media were used, both of which were supplemented with testosterone in order to elucidate the question whether the bone-derived MSCs are able to synthesize 17β-estradiol by aromatase (CYP19A1) during differentiation. This approach has shown that significantly high levels of 17β-estradiol (produced from testosterone) were detectable when the MSCs were cultured in the osteogenic and adipogenic media. Additionally, the cells incubated within these media (e.g. the OMT and AMT) revealed aromatase activity on the protein level as shown by immunofluorescence (Fig. 2) confirming the finding that estrogen can be produced locally in differentiated human MSCs by aromatase (Nelson and Bulun, 2001; Pino et al., 2006). Regarding the MSCs cultivated within the basal media (BM), it could be shown that irrespective of further supplementation (BMT or BMTA) neither considerable amounts of 17β-estradiol nor aromatase activity were detectable thereby indicating that detectable aromatase expression and activity is strictly limited to differentiated cells. This is consistent with Leskelä et al. (2006) who reported the absence of aromatase activity in bone marrow derived hMSCs under standard culture conditions in the presence of testosterone.

As the use of anastrozole not only abrogated 17β-estradiol synthesis (Fig.1, OMTA and AMTA) but also distinctly changed the pattern of gene expression (Fig. 9), it can be concluded that the progression of osteogenesis was markedly influenced by 17β-estradiol. These findings contrast to the results of Hong et al. (2006) who found an increase of both, the osteogenic and the adipogenic differentiation capabilities of MSCs, in response to 17β-estradiol. However, in accordance with the present results, documenting a significant increase of *Runx-2*, *BMP-2*, and *Cx43* as well as a slight increase in *OCN* expression, Ray et al. described stimulation of osteogenesis of cultured human bone marrow derived MSCs in response to 17β-estradiol (Ray et al., 2008). These authors reported that BMP-2 and osteocalcin expressions were stimulated, and – as shown presently – that calcium deposition of the cultures was increased. The calcium deposition is an indicator for the late stage of osteogenic differentiation. Alizarin staining of hMSCs in the presented study indicated a significant increase of this late stage of osteogenic differentiation process in the presence of 17β-estradiol (OMT, Fig. 5). An improvement of late osteogenic differentiation by 17β-estradiol has been described in the literature (Hong et al., 2006; Ray et al., 2008). In rat bone-marrow derived MSCs the evidence of bone nodule formation (by means of alizarin staining) significantly increased with E2-treatment (Song et al., 2017). Similarly, estrogen is important in the late phase of osteogenic differentiation and osteoblast maturation in MC3T3-E1 cells (Kubota et al., 2018). Also testosterone is able to influence cells during the mineralization stage of osteogenic differentiation (Wiren et al., 2002) and affects mature osteoblasts (Syed and Khosla, 2005). This is in line with the presented results regarding hMSCs cultivated in OMTA with significantly reduced alizarin staining compared to OM (Fig. 5). The literature analysis of an estradiol effect on the early stages of osteogenic differentiation differed from our results showing no significant influence of E2 (OMT) on ALP activity (Fig. 6). In human as well as rat bone marrow derived MSCs, an increase of ALP after E2-treatment is described (Niada et al., 2016; Song et al., 2017). However, the cultivation times differed from ours in both studies (21 days in the presented study vs. 14 days (Niada et al., 2016) vs. 7 days (Song et al., 2017)). Furthermore, other cell sources as in the presented study are used and it is known that different cells demonstrate various responses to E2 (Niada et al., 2016).

Runx-2 is a transcription factor expressed in the course of osteogenic differentiation (Kawai et al., 2012), and BMP-2 induces osteogenic differentiation of MSCs by a Runx-2-dependent pathway (Pino et al., 2012). Additionally, Runx-2 is a key target of the gap junction protein Cx43 (Stains and Civitelli, 2016) representing the most common connexin in cells of the bone. Cx43 is required for cell differentiation and function (Plotkin and Bellido, 2013; Stains and Civitelli, 2016), and according to this, it has been demonstrated that Cx43 expression is upregulated during osteogenesis of human bone-derived MSCs *in vitro* along with bone sialoprotein expression and enhanced matrix mineralization (Wagner et al., 2017). Moreover, a sex-dependent variability concerning the response to different estrogen levels has been described for MSCs of rats and humans (Hong et al., 2009; Leskelä et al., 2006).

17β-estradiol mediates growth and differentiation of MSCs through the ERα receptor (Ray et al., 2008). ERα upregulation in MSCs of osteoporotic mice has been reported in response to 17β-estradiol whereas ERβ expression was decreased (Zhou et al., 2001). Although the structurally close related ERs exhibit similar expression patterns and possess a widely overlapping spectrum of target genes, in bone tissue they act differently and ERβ has inhibitory effects on ERα-induced bone growth (Bado et al., 2017). However, no information is currently available defining detailed functional differences between the estrogen receptor isoforms (Patel et al., 2018). Therefore, their action on bone is still not understood completely (Khosla and Monroe, 2018). Regarding

the present results of an undetectable *ERβ*-mRNA expression in human bone-derived MSCs it has been taken into consideration that the donors were postmenopausal women with a mean age of 72.6 years. It has been reported that the expression of estrogen receptors in bone cells is age-dependent (Bado et al., 2017; Batra et al., 2003). In humans younger than age 40 both isoforms, ERα and ERβ, are expressed in cells of the bone. However, in postmenopausal women the expression of ERβ decreased in osteoblasts and mesenchymal stromal cells while ERα is constantly expressed (Batra et al., 2003). Moreover, ERα – and not ERβ – is associated with differentiation processes of osteoblasts (Khalid and Krum, 2016), and during osteogenic differentiation of rat calvarial cells *in vitro* only ERα expression increases in the course of matrix maturation (Wiren et al., 2002). Accordingly, as summarized in a recent review, especially ERα signalling plays a major role in mature osteoblasts of mice (Khosla and Monroe, 2018).

Our study further showed, that the expression of *PPARγ*, representing an important transcription factor and nuclear receptor involved in adipogenesis (Kawai et al., 2012), was significantly reduced over the time in cells cultured in AMT and AMTA. Accordingly, cells containing lipid droplets were preferentially seen in the AM, and rarer within the cytoplasm of cells cultured in AMT and AMTA (Fig. 6). In accordance with our observation, testosterone was shown to inhibit adipogenic differentiation of a 3T3-L1 cell line (Singh et al., 2006), and to reduce proliferation of adipocytes (Ray et al., 2008). Moreover, dihydrotestosterone (DHT) inhibited adipogenesis of human MSCs (Gupta et al., 2008; Russell et al., 2018). Considering the relationship between AR receptor expression and adipogenesis, it has been reported that the AR is downregulated in human MSCs by DHT under adipogenic culture conditions (Gupta et al., 2008).

Accordingly, adipogenic differentiation and the significantly reduced expression pattern of *PPARγ* were accompanied by significantly lower *AR* expression levels, concomitantly with undetectable *ERα* when the cells were cultured in AMT and AMTA for 21 days. Considering *AR,* it was concluded that its expression was slightly increased (compared to AMT without reaching significant levels) by the high testosterone level predominating in AMTA. The regulatory mechanism underlying the similar expression pattern of *AR* and *PPARγ* in AMT and AMTA is less clear, as both steroids were present in biologically active concentrations with E2 in AMT and testosterone in AMTA respectively. It has been reported that nongenotropic, non-apoptotic effects of sex steroids on osteoblasts are mediated by different sex steroid receptors (e.g. ERα, ERβ, and AR) with similar efficiency notwithstanding whether the ligand is androgen or estrogen (Kousteni et al., 2001). Finally, it might be concluded that remaining, still not enzymatically converted levels of testosterone within the AMT might be responsible for the expression of this receptor.

5. Conclusions

17β-estradiol-mediated osteogenic effects on MSCs depend on aromatase activity of the human bone-derived MSCs and are limited to differentiated cells of the osteogenic cultures expressing ERα. Suppression of adipogenesis of the cells is induced by both testosterone and 17β-estradiol. Therefore, it might be concluded that changes of sex steroid levels due to old age modulate differentiation capacity of the bone-derived MSCs in terms of reducing osteogenesis and increasing adipogenesis, resulting in age related bone loss and excessive increase of bone marrow fat.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Kristina Glenske: Conceptualization, Investigation, Methodology, Visualization, Writing - original draft. **Gerhard Schuler:** Conceptualization, Investigation, Methodology, Writing - original draft. **Stefan Arnhold:** Resources, Writing - review & editing. **Mohamed I. Elashry:** Resources. **Alena-Svenja Wagner:** Visualization. **Mike Barbeck:** Resources, Writing - review & editing. **Elena Neumann:** Resources, Writing - review & editing. **Ulf Müller-Ladner:** Resources. **Reinhard Schnettler:** Conceptualization, Supervision. **Sabine Wenisch:** Conceptualization, Project administration, Supervision, Writing - original draft.

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