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The Phytoestrogen Genistein Enhances Osteogenesis and Represses Adipogenic Differentiation of Human Primary Bone Marrow Stromal Cells

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In the present study, we investigated the role of the phytoestrogen genistein and 17β-estradiol in human bone marrow stromal cells, undergoing induced osteogenic or adipogenic differentiation. Profiling of estrogen receptors (ERs)-α, -β1, -β2, -β3, -β4, -β5, and aromatase mRNAs revealed lineage-dependent expression patterns. During osteogenic differentiation, the osteoblast-determining core binding factor-α1 showed a progressive increase, whereas the adipogenic regulator peroxisome proliferator-activated receptor γ (PPARγ) was sequentially decreased. This temporal regulation of lineage-determining marker genes was strongly enhanced by genistein during the early osteogenic phase. Moreover, genistein increased alkaline phosphatase mRNA levels and activity, the osteoprotegerin:receptor activator of nuclear factor-κB ligand gene expression ratio, and the expression of TGFβ1. During adipogenic differentiation, down-regulation in the mRNA levels of PPARγ and CCAAT/enhancer-binding protein-α at d 3 and decreased lipoprotein lipase and adipsin mRNA levels at d 21 were observed after genistein treatment. This led to a lower number of adipocytes and a reduction in the size of their lipid droplets. At d 3 of adipogenesis, TGFβ1 was strongly up-regulated by genistein in an ER-dependent manner. Blocking the TGFβ1 pathway abolished the effects of genistein on PPARγ protein levels and led to a reduction in the proliferation rate of precursor cells. Overall, genistein enhanced the commitment and differentiation of bone marrow stromal cells to the osteoblast lineage but did not influence the late osteogenic maturation markers. Adipogenic differentiation and maturation, on the other hand, were reduced by genistein (and 17β-estradiol) via an ER-dependent mechanism involving autocrine or paracrine TGFβ1 signaling. (*Endocrinology* 145: 848–859, 2004)

THE ESTROGEN RECEPTOR (ER)α, and the more recently discovered ERβ, belong to the nuclear hormone receptor superfamily. They mediate a variety of physiological signals (1) by directly activating and/or repressing gene transcription (2). The soy-derived isoflavone genistein shares structural similarities with the hormone 17β-estradiol (E2). In competition binding experiments, genistein binds to both receptors but with a higher relative binding affinity for ERβ1 (3). Genistein has also been shown to transactivate estrogen receptor response element-driven reporter gene expression by both the human ERα and ERβ1 (4). Genistein is, therefore, referred to as a phytoestrogen.

Estrogens are important in the maintenance of human bone homeostasis (5, 6). After menopause, bone turnover is enhanced due to a lack of endogenous E2. This increase is accompanied by a dramatic shift in the adipocyte:osteoblast ratio toward the direction of fat tissue production in the bone marrow (7, 8). However, cells of the adipocyte and osteoblast lineages have been shown to exhibit high plasticity and the potential for interconversion (9, 10). Moreover, there is evidence that endogenous estrogen production by Cyp19 aromatase as well as ER signaling play an important role in the development and distribution of white adipose tissue in the body. This result was recently highlighted by an analysis of the respective ERα and Cyp19 knockout mice (11, 12).

The core binding factor α1 (Cbfa-1), belonging to the runt domain transcription factor family, is an essential mediator for launching osteoblastogenesis in mesenchymal progenitor cells (13). Commitment to adipogenesis is initiated by the peroxisome proliferator-activated receptor γ (PPARγ) together with the CCAAT/enhancer binding proteins (C/EBPs). They act by synergistically transactivating the expression of several adipogenic effector genes (14, 15).

Genistein has been demonstrated to augment lipogenesis and lipolysis in isolated rat adipocytes (16). Recent data have reported that E2 can modulate the adipogenic commitment of the mesenchymal mouse cell line KS483 via transcriptional repression of PPARγ (17). E2 also favored early osteogenic commitment and inhibited adipogenesis of mouse ST2 cells overexpressing either ERα or -β (18). Harmon *et al.* (19) reported that genistein can down-regulate the expression of

Abbreviations: ALP, Alkaline phosphatase; AM, adipogenic medium; BMSC, bone marrow stromal cell; BrdU, 5-bromo-2′-deoxyuridine; BSP, bone sialoprotein; Cbfa-1, core binding factor α1; C/EBP, CCAAT/enhancer binding protein; Ct, cycle threshold; ΔCt, $Ct_{target} - Ct_{18S\ RNA}$; $\Delta\Delta Ct_{treatment}$, $\Delta Ct_{treatment} - \Delta Ct_{d\ 0\ control}$; E2, 17β-estradiol; ER, estrogen receptor; FCS, fetal calf serum; LPL, lipoprotein lipase; mAb, monoclonal antibody; OM, osteogenic differentiation medium; OP, osteopontin; OPG, osteoprotegerin; PPARγ, peroxisome proliferator-activated receptor γ; RANKL, receptor activator of nuclear factor-κB ligand.

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PPARγ and C/EBPα in 3T3-L1 cells by inhibiting C/EBPβ activity. It does so by increasing levels of the dominant negative inhibitory C/EBP homologous protein CHOP/GADD153.

The interaction between the estrogenic and adipogenic pathways and the role of the natural food ingredient genistein on the inverse relationship between bone marrow adipogenesis and osteoblastogenesis require further clarification. We approached this problem using human bone marrow stromal cell (BMSC) cultures. These cells have been shown to contain pluripotent mesenchymal progenitor cells that can give rise to several lineages such as osteoblasts, adipocytes, chondrocytes, and myoblasts (20, 21). This study focused on investigating the effects of physiologically relevant concentrations of genistein that can be achieved by nutritional intake or supplementation in the differentiation and maturation of human osteoblasts and adipocytes from BMSCs.

Materials and Methods

Reagents

Chemically synthesized genistein (5,7,4′-trihydroxyisoflavone) was kindly provided by Dr. D. Burdick (Roche Vitamins Ltd., Basel, Switzerland) and 17β-estradiol was received from Sigma-Aldrich (Buchs, Switzerland). The pure antiestrogen ICI 182,780 was purchased from Tocris (Bristol, UK). The mouse anti-hTGFβ1 monoclonal antibody (clone 9016.2) was obtained from R&D Systems (Abingdon, UK). Cell culture reagents and PCR primers were purchased from Invitrogen (Basel, Switzerland) and TaqMan probes were ordered from Integrated DNA Technologies (IDT, Coralville, IA) and Applied Biosystems (Rotkreuz, Switzerland). If not indicated otherwise, additional reagents were obtained from Sigma-Aldrich at molecular biology grade.

Bone marrow stromal cell isolation, expansion, and culture

Bone marrow aspirates from healthy perimenopausal female donors (41–51 yr) were obtained from iliac crest biopsies, during routine surgical procedures, after informed consent and the approval of the local ethical commission. Mononucleated cells were isolated by density gradient centrifugation using Histopaque solution (Sigma) and subsequently seeded and expanded in DMEM (4.5 mg/ml D-glucose), 10% fetal calf serum (FCS), 100 μM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine supplemented with 10 nm dexamethasone and 5 ng/ml fibroblast growth factor-2 (R&D Systems, Wiesbaden, Germany), as previously described (22).

Osteogenic stimulation

At subconfluency, cells were detached with trypsin/EDTA and plated in 6-well cell culture dishes at a density of 3000 cells/cm^2. Cells were then cultured in osteogenic differentiation medium (OM), consisting of DMEM plus 10% charcoal-treated FCS supplemented with 10 nM dexamethasone, 100 μM L-ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate for up to 18 d. Treatments consisted of either vehicle (0.1% dimethylsulfoxide), 10 nM genistein, 1 μM genistein, or 10 nM E2. Medium and treatments were changed twice weekly. For biochemical and mRNA analyses, cells were harvested at timed intervals (3, 7, 11, 15, and 18 d).

Adipogenic stimulation

For adipogenic differentiation, cells were seeded at the same density as above and cultured in DMEM, 1% charcoal-treated FCS (low serum) supplemented with 100 nM dexamethasone and 10 nM insulin [adipogenic medium (AM)] for up to 21 d. mRNA analysis and biochemical assays were performed on d 3 and 21. Treatments were the same as those described for the osteogenic stimulation. In inhibition experiments, 5 μM ICI 182,780, 5 μg/ml anti-hTGFβ1, or a mouse IgG isotype control (sc-2025, Santa Cruz Biotechnology, Santa Cruz , CA) was added before the estrogenic compounds.

Quantitative real-time RT-PCR

For quantification of gene expression levels, we used a real-time TaqMan RT-PCR assay based on the multiplex method as previously described (23). Briefly, total RNA was isolated using the RNeasy minikit with an on-column DNase I digest (Qiagen, Basel, Switzerland). First-strand cDNA was synthesized in a 20-μl reaction from 5 μg total RNA by reverse transcription with SuperScript II (Invitrogen) using 250 ng random hexamers (Microsynth, Balgach, Switzerland). The conditions for the cDNA synthesis were: 10 min for random primer annealing at 70 C followed by a 2-min cooling step and then the reverse transcription reaction (25 C for 10 min, 42 C for 30 min, 48 C for 20 min, and 70 C for 15 min). The cDNA was subsequently diluted to 500 μl in diethylpyrocarbonate-treated water. In a 50-μl PCR, 10 μl cDNA (corresponding to 100 ng total RNA input) were amplified in a 7700 sequence detector (Applied Biosystems), using the 2 × Universal master mix (Applied Biosystems), 25 nM primers, and 100 nM probe (VIC-TAMRA labeled) for the 18S rRNA internal control, and 300 nM primers and 200 nM probe (FAM-TAMRA labeled) for the gene of interest.

All probe and primer sets were designed with the Primer Express program version 1.0 (Applied Biosystems) and initially tested to have comparable (>90%) efficiency in multiplex assays using 18S rRNA as internal control. TaqMan probe and primer oligonucleotide sequences are as follows: 18S rRNA: forward primer 5′-CGGCTACCACATCCAAGGAA-3′, reverse primer 5′-GCTGGAATTACCGCGGCT-3, probe 5′-VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA-3′; alkaline phosphatase (ALP): forward primer 5′-GACCCTTGACCCCCACAAT-3′, reverse primer 5′-GCTCGTACTGCATGTCCCCT-3′, probe 5′-FAM-TGGACTACCTATTGGGTCTCTTCGAGCCA-TAMRA-3′; TGFβ1: forward primer 5′-AAGGGCTACCATGCCAACTTC-3′, reverse primer 5′-TGCGTGTCCAGGCTCCA-3′, probe 5′-FAM-CCTCGGGCCCTGCCCCTACA-TAMRA-3′; Cbfa-1: forward primer 5′-GCCTTCAAGGTGGTAGCCC-3′, reverse primer 5′-CGTTACCCGCCATGACAGTA-3′, probe 5′-FAM-CCACAGTCCCATCTGGTACCTCTCCG-TAMRA-3′; bone sialoprotein (BSP)-2: forward primer 5′-TGCCTTGAGCCTGCTTCC-3′, reverse primer 5′-GCAAAATTAAAGCAGTCTTCATTTTG-3′, probe 5′-FAM-CTCCAGGACTGCCAGAGGAAGCAATCA-TAMRA-3′; osteoprotegerin (OPG): forward primer 5′-ACCACAATGAACAAGTTGCTGTG-3′, reverse primer 5′-CTGGGTGGTCCACTTAATGGA-3′, probe 5′-FAM-TGTCCAGAAACACGAGCGCGCA-TAMRA-3′; receptor activator of nuclear factor-κB ligand (RANKL): forward primer 5′-CGTTGGATCACAGCACATCAG-3′, reverse primer 5′-TGCTCCTCTTGGCCAGATCT-3′, probe 5′-FAM-CAGAGAAAGCGATGGTGGATGGCTCAT-TAMRA-3′; PPARγ: forward primer 5′-TCAGAAATGCCTTGCAGTGG-3′, reverse primer 5′-TTCTCGGCCTGTGGCATC-3′, probe 5′-FAM-TGTCTCATAATGCCATCAGGTTTGGGC-TAMRA-3′; C/EBPα: forward primer 5′-AAGAAGTCGGTGGACAAGAACAG-3′, reverse primer 5′-TGCGCACCGCGATGT-3′, probe 5′-FAM-AACGAGTACCGGGTGCGGCG-TAMRA-3′; lipoprotein lipase (LPL): forward primer 5′-CAGCAAAACCTTCATGGTGAT-3′, reverse primer 5′-CAAGTTTTGGCACCCAACTC-3′, probe 5′-FAM-CATACATTCCTGTTACCGTCCAGCCAT-TAMRA-3′; Adipsin: forward primer 5′-TCCTGGCCTAGGGTGCC-3′, reverse primer 5′-GATGCAGGAGTGGATGACTTCA-3′, probe 5′-FAM-CTGAAGGTCAGGGTCACCCAAGCAA-TAMRA-3′; ERα: forward primer 5′-GGCTGGCCCAGCTCCT-3′, reverse primer 5′-CAGATGCTCCATGCCTTTGTT-3′, probe 5′-FAM-CATCCTCTCCCACATCAGGCACATGA-TAMRA-3′; ERβ1: forward primer 5′-CCTGGCTAACCTCCTGATGCT-3′, reverse primer 5′-CAGATGTTCCATGCCCTTGTT-3′; probe 5′-FAM-TGTCCCACGTCAGGCATGCGA-TAMRA-3′; ERβ2: forward primer 5′-CCTGGCTAACCTCCTGATGCT-3′, reverse primer 5′-TCTTTGAGAGGCCTTTTCTGC-3′; probe 5′-FAM-TGTCCCACGTCAGGCATGCGA-TAMRA-3′; ERβ3: forward primer 5′-CCTGGCTAACCTCCTGATGCT-3′, reverse primer 5′-AATCTCCAAGAGAGTGAGAGAGAGCT-3′, probe 5′-FAM-TGTCCCACGTCAGGCATGCGA-TAMRA-3′; ERβ4: forward primer 5′-CCTGGCTAACCTCCTGATGCT-3′, reverse primer 5′-TGAATTGCTTTTCTCCCCATCT-3′, probe 5′-FAM-TGTCCCACGTCAGGCATGCGA-TAMRA-3′; ERβ5: forward primer 5′-ATCCATGCGCCTGGCTAAC-3′, reverse primer 5′-CTCCTTAGGGCGCGTACCT-3′, probe 5′-FAM-TGTCCCACGTCAGGCATGCGA-TAMRA-3′; Cyp19 aromatase:

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forward primer 5′-TGGTGTGGAATTATGAGGGCA-3′, reverse primer 5′-GCCGTGGGAGATGAGGG-3′, probe 5′-FAM-TCCTCAATACCAGGTC-CTGGCTACTGCATG-TAMRA-3′.

Relative gene expression levels are presented as $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{18S\ RNA}$ and the Ct value is the cycle threshold. The SD was determined from at least three independent experiments of the ΔCt values. The upper and lower errors were defined as $2^{-(\Delta Ct-SD)}$ and $2^{-(\Delta Ct+SD)}$, respectively. Fold change for the treatment was defined as the relative expression, compared with the d 0 control and was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct_{treatment} = \Delta Ct_{treatment} - \Delta Ct_{d\ 0\ control}$. The ΔΔCt method was applied according to the Applied Biosystems user bulletin #2 (ABI Prism 7700 Sequence detection system).

Staining of mature adipocytes

Fat droplets within differentiated adipocytes were observed at d 21 using the Oil Red O staining method as previously described (23). Briefly, cells were fixed with 60% isopropanol, and, after washing with 1 × PBS, a dilution of 0.25% (wt/vol) Oil Red O (Sigma) in 60% isopropanol was added for 20 min. Photomicrographs were taken with a digital IX 50 microscope (Olympus, Tokyo, Japan) at ×20 and ×40 magnifications. The number of adipocytes was calculated by counting Oil Red O-positive cells in 16 separate fields, using three wells per condition.

Cellular alkaline phosphatase activity

Cells were rinsed twice with 1 × PBS and then lysed in 50 mM Tris-HCl (pH 7.4), 0.2 mM $MgCl_2$, and 0.02% Triton X-100 at 4 C. Subsequently, cells were sonicated for 1 min and centrifuged at 10,000 rpm in an Eppendorf centrifuge. Alkaline phosphatase activity was measured, in triplicate, as the rate of conversion of *p*-nitrophenyl-phosphate using the Sigma kit 104–40 as described by the manufacturer. Total protein was then measured using the BCA kit (Pierce, Rockford, IL). Alkaline phosphatase activity is expressed as specific activity (micromoles nitrophenol per hour per mg total protein).

Cell proliferation assay

To determine the proliferation rate of stromal/preadipocyte cells at d 3 in adipogenic differentiation medium, we applied a colorimetric 5-bromo-2′-deoxyuridine (BrdU) ELISA (Roche Biochemicals, Rotkreuz, Switzerland). Cells were initially seeded at a density of 1×10^4 cells/well in 24-well plates using the media and supplements as described in *Adipogenic stimulation*. For quantitative determination, the cells were pulsed for 18 h with the BrdU reagent and subsequently treated as described by the manufacturer.

Western blotting

At d 3 of adipogenic stimulation, total cell protein was harvested in NETT-C buffer [100 μM NaCl, 1 mM EDTA, 10 mM Tris (pH 7.5), 1% Triton X-100, and Complete protease inhibitors (Roche Biochemicals)] at 4 C. After 1-min sonication, lysates were cleared by centrifugation at 10,000 rpm for 15 min, and the supernatant protein content was quantified. Twenty micrograms of cleared extract were resolved under reducing conditions by 10% Tris-glycine SDS-PAGE and consequently electrotransferred to an Immobilon-P polyvinyl difluoride membrane (Millipore, Bedford, MA) for Western analysis. The membranes were blocked with 2.5% Western blocking reagent (Roche Biochemicals), plus 2.5% FCS, overnight at 4 C in PBS plus Tween 20. Immunoblotting was performed using a 1:700 dilution of a rabbit anti-PPARγ polyclonal Ab (H-100, Santa Cruz Biotechnology) with an antiactin monoclonal antibody (mAb) (Sigma-Aldrich) at a concentration of 1:2000 for 1 h as internal control. Horseradish peroxidase-labeled anti-IgGs (Amersham Biosciences, Dübendorf, Switzerland) were used as secondary antibodies in a 1:5000 dilution and chemiluminescence was measured using ECL Plus reagents (Amersham Biosciences).

Statistical analysis

Statistics were assessed using a *t* test, assuming double-sided independent variance, and $P < 0.05$ was considered significant. Significance levels for the TaqMan data were assessed at the stage of ΔΔCt values. The upper and lower limits of mRNA expression levels were calculated as described above. All other values are presented as mean ± SD.

Results

Gene expression profiling revealed a switch in ER isotypes during differentiation

We profiled the mRNA expression levels of the ERα and -β receptor isoforms, including the splice variants β1, β2, β3, β4, and β5 as well as Cyp19 aromatase to characterize estrogenic signaling during osteoblastic differentiation (at d 0, 3, 7, 11, 15, and 18) and adipogenic differentiation (at d 0, 3, and 21) of human BMSCs. To obtain the relative gene expression values of the receptors and cyp19 aromatase, we applied multiplex quantitative real-time RT-PCR. ERα mRNA levels were first detected in undifferentiated cultures at d 0 and gradually increased during both osteoblastic and adipogenic differentiation. The highest expression levels occurred at the final time points at d 18 and 21, respectively (Fig. 1A). ERβ1 and ERβ2 were found to be expressed at low levels in BMSCs, and these levels increased during osteogenesis but remained low during adipogenesis. ERβ3 was not detected during either osteogenic or adipogenic maturation. In the undifferentiated stromal cells, ERβ4 mRNA was the most abundant ER transcript but decreased to undetectable levels during both differentiation processes. We also observed that ERβ5 mRNA expression levels were substantially up-regulated during osteogenesis, but they decreased to undetectable levels during adipogenesis. During osteogenic differentiation, we found a steady increase of Cyp19 aromatase until d 18, whereas after a sharp increase at d 3 of adipogenesis, the expression dropped to extremely low levels at d 21. Interestingly, the ER and Cyp19 expression patterns from the precursor BMSCs changed during maturation toward two new, but different, expression profiles for either osteoblastogenesis or adipogenesis (Fig. 1B).

Genistein stimulated genes for osteogenic differentiation

BMSC cultures from five different perimenopausal donors were expanded and subsequently differentiated using osteogenic supplements. To measure the effects of physiologically high (1 μM) and low (10 nM) genistein concentrations, as well as E2 (10 nM), on osteoblast commitment and maturation in a time-dependent manner, the expression of several osteoblast-related and stage-dependent marker genes were quantified using multiplex quantitative real-time RT-PCR assays. The genes analyzed included the osteoblast differentiation markers ALP, osteopontin (OP), BSP-2, osteocalcin, and the osteoblast transcription factor Cbfa-1/Runx-2. Factors that have been shown to be mediators of osteoblast/osteoclast coupling, such as RANKL/osteoclast differentiating factor, its decoy receptor OPG/osteoclast inhibiting factor, and TGFβ1, were also analyzed. In addition, the expressional behavior of the early adipogenic transcription factors PPARγ and C/EBPα was investigated during osteogenesis. There was high variance between the baseline expression levels of some marker genes in the cultures of individual donors. This has recently been reported by our group (22). We were, nevertheless, able to detect consistent

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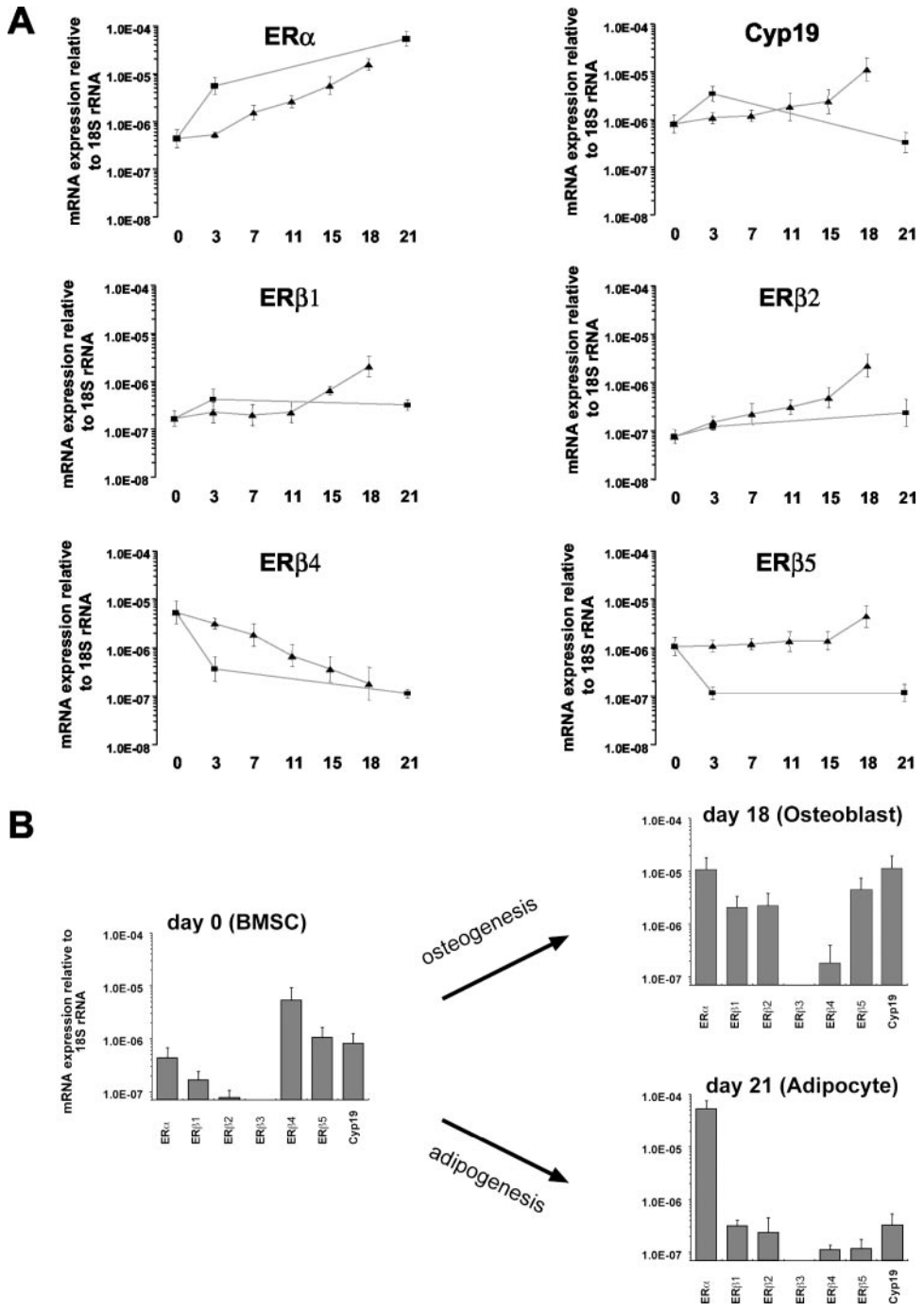

FIG. 1. ERα, -β1, -β2, -β4, and -β5 and Cyp19 aromatase mRNA expression levels were assessed by quantitative real-time RT-PCR. The time course for ERα, -β1, -β2, -β4, and -β5 and Cyp19 aromatase mRNA expression during osteogenic and adipogenic differentiation of BMSCs is shown (A). BMSC cultures from perimenopausal donors were expanded and subsequently differentiated into either the osteogenic (▲) (five donors) or the adipogenic lineage (■) (three donors). Each BMSC culture was analyzed in triplicate, and the average of the combined results of all donors is presented. Total RNA was harvested at timed intervals and prepared for multiplex quantitative real-time RT-PCR analysis. Gene expression levels normalized to 18S rRNA were calculated from at least three independent experiments and are presented as $2^{(-\Delta Ct)}$. During differentiation gene expression of ERα, Cyp19 aromatase, ERβ1, ERβ2, ERβ4, and ERβ5 was detected. ERβ3 was not present. A comparison of the relative gene expression profiles of BMSCs at d 0 (B, left panel) and after differentiation either to osteoblasts at d 18 or to adipocytes at d 21 is represented (B, right panels). ERα, β1, β2, β4, and β5 and Cyp19 aromatase mRNA levels were determined as described above. Expression levels less than 1.0E-07 relative to 18S rRNA were marginally low and therefore considered not to be present by definition.

gene expression changes after estrogenic treatment after applying a correction for the relative basal mRNA expression levels.

The mRNA expression of ALP was found to peak at d 11, but decreased at d 15–18 of differentiation (Fig. 2A). The observed decrease in the vehicle-treated group was partially counteracted by treatment with 1 μM genistein or 10 nM E2. Moreover, we were able to demonstrate that ALP protein activity, measured at d 18 in OM, showed a significant increase in those cultures treated with 1 μM genistein or 10 nM E2. The use of a lower concentration of genistein (10 nM) was not effective in up-regulating ALP protein activity (Fig. 2B), which paralleled the observations obtained with quantitative real-time RT-PCR.

We have recently shown that the extracellular matrix genes BSP-2 and OP increase significantly during osteogenic differentiation of BMSCs (22). However, we were not able to observe a relevant change in BSP-2 by the different estrogenic treatments. Also, only a slight trend toward up-regulation was observed for OP mRNA at d 15 and 18 in the E2-treated cultures. The classical osteoblast maturation marker osteocalcin showed high variability among cultures from different donors. There was no significant effect after estrogenic treatment (data not shown).

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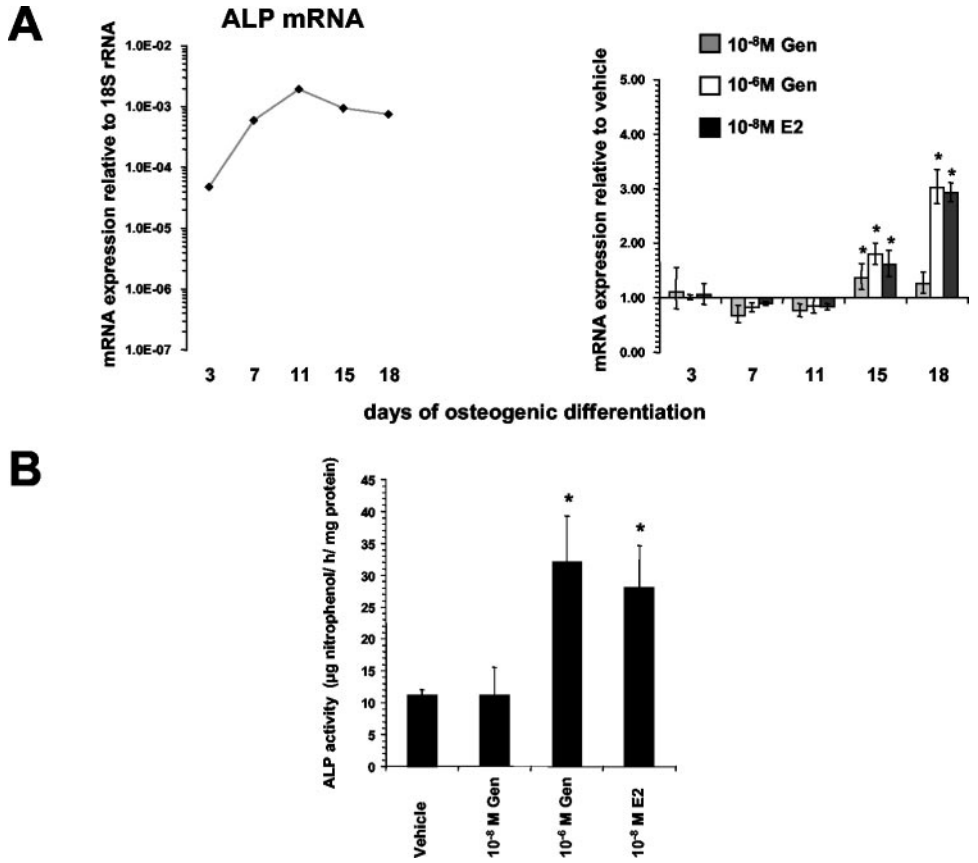

FIG. 2. Genistein enhances ALP expression and activity during osteogenic differentiation. BMSCs were grown in OM for up to 18 d and mRNA expression levels of ALP were determined using quantitative real-time RT-PCR after treatment (A). The ALP mRNA values normalized to 18S rRNA during osteogenic differentiation for the vehicle-treated control are shown by ◆ symbols (A, left panel), whereas the fold changes induced by 10^{-8} M genistein (Gen, *gray*), 10^{-6} M genistein (*white*), and 10^{-8} M E2 (*black*) are indicated by *bars* (A, right panel). The gene expression data represent the mean from no less than five experiments. The values from the treatment groups are shown with +/− upper and lower limits; unpaired *t* test; *, $P < 0.05$. Normalized ALP protein activity was determined using the *p*-nitrophenyl-phosphate method (B). At d 18 of osteogenic differentiation, a significant increase for 10^{-6} M genistein and 10^{-8} M E2 was measured. Values represent the mean ± SD; *, $P < 0.05$.

The mRNA levels of RANKL progressively decreased during the differentiation process (Fig. 3) and were not affected by any of the estrogenic treatments. In contrast, the mRNA levels of OPG were observed to increase gradually during osteogenesis (Fig. 3), and this effect was further enhanced by supplementation with either genistein at 10 nM or 1 μM or with 10 nM E2 starting at d 7. Thus, osteogenic differentiation led to a net increase in the OPG:RANKL ratio, which was further enhanced by genistein or E2. The increase became more prominent toward the mineralization phase. Notably, the expression of TGFβ1 increased during osteogenesis (Fig. 3) and was strongly up-regulated by genistein and E2 treatment.

Calcified nodule formation became apparent beginning at d 15 of osteogenic stimulation; thus, we examined the effects of genistein and E2 on the accumulation of HCl-extractable calcium at d 18. No significant change was observed by any of the treatments (data not shown).

We were also able to demonstrate an inverse regulation between increasing Cbfa-1 and decreasing PPARγ mRNAs during osteogenesis (Fig. 3). This could be further enhanced by stimulation of the cells with 1 μM genistein or 10 nM E2, especially at early time points between d 3 and 7.

Genistein treatment repressed early and late adipogenic differentiation markers

Initial observations that showed PPARγ expressional down-regulation by genistein and E2 during osteogenesis led us to investigate the role of genistein during adipogenesis. Adipogenesis was induced in BMSC cultures using AM, as previously described (24).

We first characterized the expression of the early adipogenic transcription factors PPARγ and C/EBPα as well as the transcription of LPL and adipsin (a marker for mature adipocytes), at d 3 and 21 after AM induction in vehicle-treated cells (Fig. 4). An increase in the relative mRNA expression levels was seen, on average, for PPARγ of 7.1-fold and 4.1-fold for C/EBPα at d 3. At d 21 this increase was slightly lower (3.6-fold for PPARγ and 2.7-fold for C/EBPα). Fold inductions were predominantly observed at d 21 for LPL (average 156.5-fold) and for adipsin (5.9-fold). We were interested whether genistein or E2 treatment could affect the mRNA levels of these genes. As shown in Fig. 4, 10 nM E2 and 1 μM genistein were sufficient in reducing the stimulatory effect of AM on PPARγ. At d 3 the PPARγ increase was reduced from 7.1-fold down to 2.9-fold. For C/EBPα this was reduced to 2.3-fold for genistein and 2.5-fold for E2. The low genistein concentration (10 nM) exerted only minor effects on these expression levels at d 3. At d 21 of adipogenic stimulation, LPL expression was reduced to 5.8-fold by 10 nM E2 and 11.9-fold by 1 μM genistein, whereas estrogenic treatment had only minor effects on adipsin mRNA levels.

Cells were stained with Oil Red O at d 21 to examine neutral fat deposition. When compared with vehicle-treated cultures (316 ± 22 adipocytes/well) (Fig 5A), we were able

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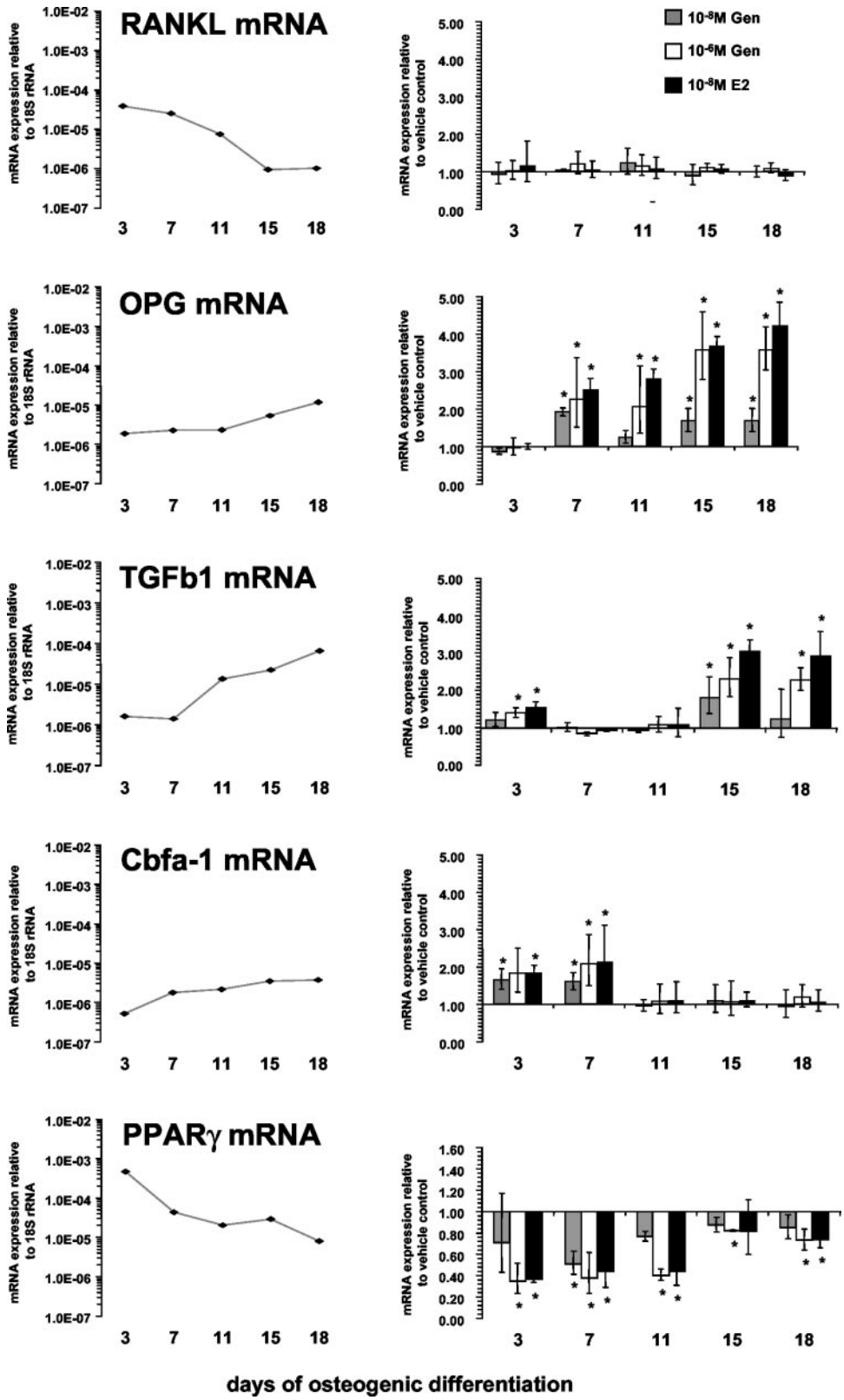

FIG. 3. Genistein enhances osteogenic gene expression. The mRNA expression levels of RANKL, OPG, TGFβ1, Cbfa-1, and PPARγ were determined during osteogenic differentiation in vehicle-treated cultures (*left panels*) and after treatment (*right panels*) with 10^{-8} M genistein (Gen, *gray bars*), 10^{-6} M genistein (*white bars*), or 10^{-8} M E2 (*black bars*). Gene expression data represent the mean of three independent experiments using cells from five individual donors (n = 15). During osteogenic stimulation the vehicle-treated controls (*left panels*) were calculated as relative expression levels to 18S rRNA. The treatment group values (*right panels*) are given as fold change to time-matched vehicle control. *Asterisks* indicate a 95% confidence level based on an unpaired *t* test.

to detect a major decrease in lipid droplet-positive cells in the cultures treated with 1 μM genistein (52 ± 18 adipocytes/well) (Fig. 5C) and 10 nM E2 (69 ± 12 adipocytes/well) (Fig. 5D). Even 10 nM genistein treatment led to a significantly lower number of fat-accumulating cells (177 ± 24 adipocytes/well) (Fig. 5B). This reduction in the number of adipocytes on treatment with genistein and E2 was matched by a smaller size in the fat droplets on the single cell level (Fig.

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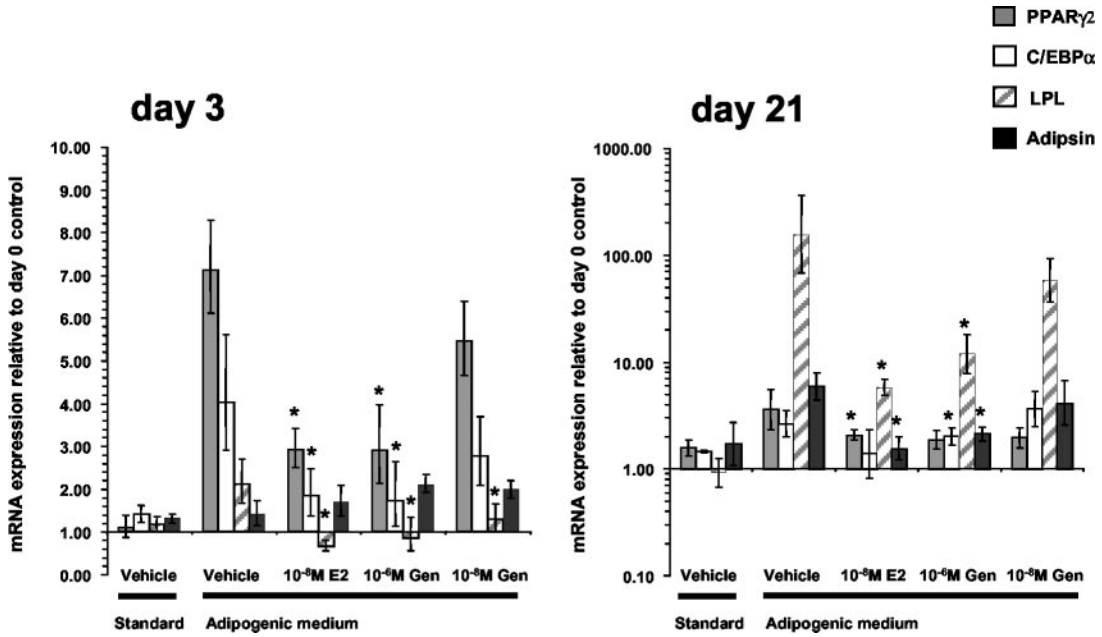

FIG. 4. Genistein inhibits adipogenic gene expression in differentiating BMSCs. Gene expression levels of adipogenic markers were determined during adipogenic stimulation using quantitative real-time RT-PCR at d 3 (*left panel*) and d 21 (*right panel*). Values were normalized to the expression levels of d 0 in which the cells were still undifferentiated. The cells of three individual donors were committed to the adipogenic lineage in triplicate (n = 9) using DMEM, 1% FCS, 10^{-8} M insulin, and 10^{-7} M dexamethasone (adipogenic medium) or were grown in DMEM, 10% FCS, vehicle (standard medium) as a negative control. Treatment with either vehicle, 10^{-8} M genistein (Gen), 10^{-6} M genistein, or 10^{-8} M E2 was performed on cells undergoing adipogenic commitment. The expression levels of the adipogenic markers PPARγ (*gray bars*), C/EBPα (*white bars*), LPL (*hatched bars*), and adipsin (*black bars*) were normalized to 18S rRNA. *Asterisks* indicate a 95% confidence level based on an unpaired *t* test.

5, *insets*). This indicates arrest at the preadipocytic differentiation state.

The antiadipogenic effects of genistein are mediated via the ER by transient autocrine or paracrine TGFβ1 signaling

During adipogenic differentiation, the basal expression levels of TGFβ1 remained unchanged. But treatment with 1 μM genistein or 10 nM E2 induced a strong and transient up-regulation of TGFβ1 mRNA at d 3 that disappeared at d 21. This up-regulation was sensitive to pretreatment with the antiestrogen ICI 182,780 (5 μM) 1 h before stimulation, showing an ER-dependent mechanism (Fig. 6, A and B). The expression levels of TGFβ1 for the control group increased at d 21 (compared with d 0), but genistein and E2 treatment had no significant impact on gene expression at this stage. Inhibition of autocrine TGFβ1 signaling at d 3, using cellular pretreatment with a neutralizing anti-TGFβ1 mAb before stimulating with the estrogenic compounds, abolished the down-regulatory effects of genistein on the protein level of PPARγ (Fig. 6C).

To detect whether up-regulated TGFβ1 had an effect on the proliferation of stromal/preadipogenic cells, we used a BrdU incorporation assay. The proliferation of cells treated with genistein or E2 was found to be significantly reduced at d 3 of adipogenesis. Adding the anti-TGFβ1 mAb abolished this effect under conditions of high and low genistein but was not able to completely abrogate the effects of E2 (Fig. 6D, *black bars*).

Discussion

To analyze the role of the phytoestrogen genistein in the differentiation and maturation of human osteoblasts and adipocytes, we used human primary BMSCs. In this study we focused on the effects of genistein on ER-mediated gene expression of lineage-determining transcription factors, as well as effector genes, during osteoblastic and adipogenic differentiation. By applying physiological concentrations of genistein, we showed that genistein can enhance the commitment and differentiation of BMSCs toward the osteoblast lineage, whereas adipogenic differentiation and maturation were hindered. Genistein lowered the number of adipocytes and decreased their lipid droplet size. Moreover, this inhibition of adipogenic differentiation could be reversed by obstructing ER and TGFβ1 signaling.

The role of the hormone E2 and the nonsteroidal soy-derived phytoestrogen genistein in the commitment and maturation of human osteoblast cells is not well defined. Therefore, we determined the mRNA profiles for ER and Cyp19 aromatase throughout all stages of osteogenic and adipogenic differentiation. These expression patterns are of interest because they reveal possible regulators of estrogen signaling during the respective differentiation states, and it should be noted that previous studies have omitted a detailed analysis of ER splice variants.

Genistein has been shown to bind to and transactivate both the ERα and the ERβ isoforms, with a preference for ERβ1 (3). However, the binding and transactivation potential for genistein on the β2-β5 splice variants (25) remains to be

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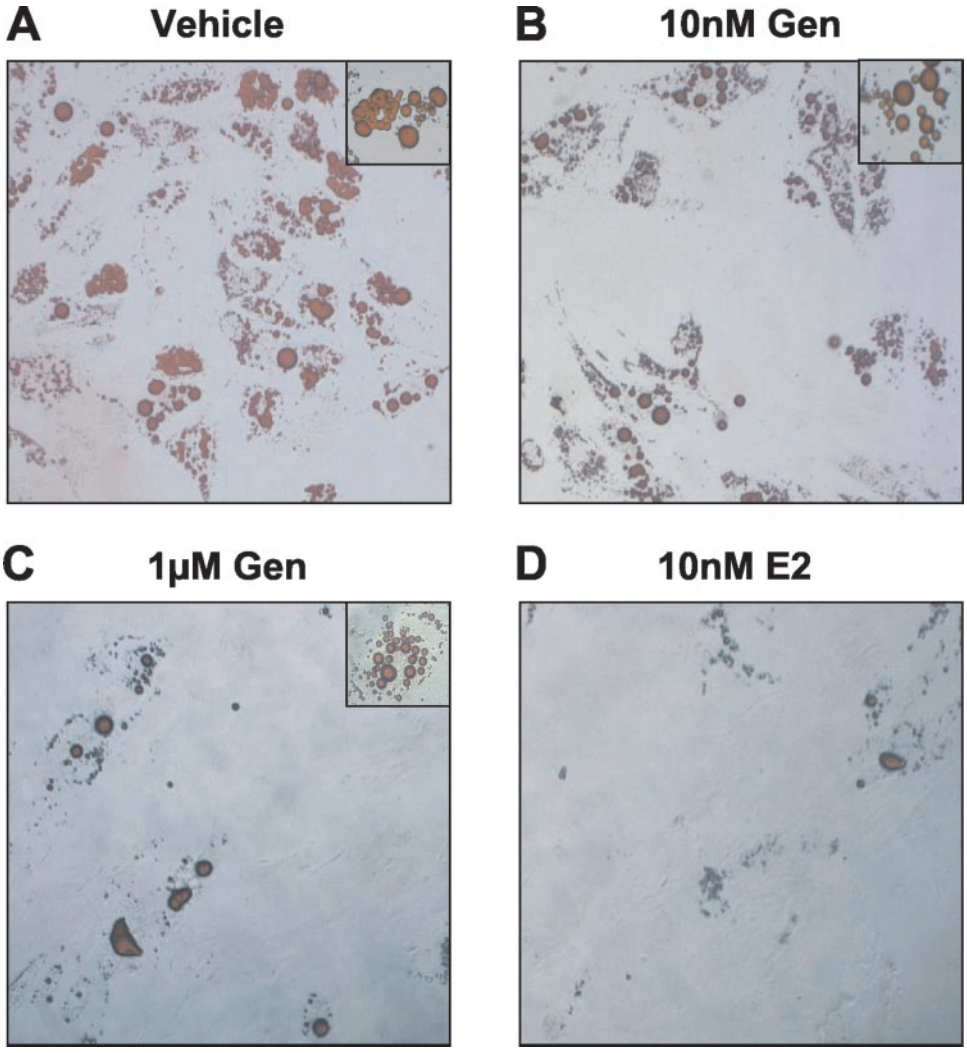

FIG. 5. Oil Red O staining of differentiated adipocytes. BMSCs were differentiated into adipocytes. Cells were then grown in 6-well plates for 21 d using 1% FCS, 10^{-8} M insulin, and 10^{-7} M dexamethasone in the absence (A) and presence of 10^{-8} M genistein (B), 10^{-6} M genistein (C), or 10^{-8} M E2 (D); for details see *Materials and Methods*. Subsequently, lipid droplets were visualized using Oil Red O staining (magnification ×20; inlays magnification ×40) and counted.

clarified. It has been suggested that the ERβ2 splice variant acts as dominant negative inhibitor of ERα transcriptional activity (26), a similar model might be the case for ERβ4 and ERβ5. As well, ERα has been reported to be the isoform predominantly expressed in mature osteoblasts (27) and adipocytes (28, 29). This implies a dominant role for ERα-mediated signaling in both cell types. We showed that the ER and Cyp19 expression patterns developed into two very different profiles over the course of BMSC differentiation into osteoblasts and adipocytes. Because this shows differential modification for estrogen signal sensitivity, it directly implies that estrogens are important in the BMSC differentiation process.

Heim, M., N. Sochocky, G. Kampmann, J. Shantz, P. Fuchs, T. Pennimpede, W. Hunziker, P. Weber, and I. Bendik (submitted manuscript) recently demonstrated that up-regulation of OPG and type 1 collagen in human trabecular bone-derived osteoblastic cells is mediated via the ERα. Consistent with these previous data, the profile from the ER isoforms in our differentiating BMSC culture model revealed a stage-dependent up-regulation of ERα in osteoblasts and adipocytes. Furthermore, the reported ER profiles of BMSCs, osteoblasts, and adipocytes (Fig. 1B) imply cell-specific differences in estrogenic sensitivity. All ERs that were already present in BMSCs were up-regulated during osteogenesis with the exception of ERβ4 that seemed to be important for the undifferentiated BMSC state. This observation is in line with the high susceptibility of estrogens in osteoblast and underlines the complex estrogenic regulation in osteoblasts that still needs to be explored. On the other side, during the adipogenic development, all ERs except ERα were down-regulated, strengthening the differences in estrogenic signaling between adipocytes and osteoblasts. Interestingly, the β5 splice variant was found to be strongly up-regulated during osteogenesis but diminished to undetectable levels in adipogenic cultures. This suggests an important role for ERβ5 in discriminating between osteogenic and adipogenic phenotypes. Thus, our data support emerging evidence that estrogens are necessary in the differentiation and distribution of white adipose tissue (12, 30).

Cyp19 aromatase is responsible for producing estrogens from C19 steroid precursors. An increase in the local production of estrogens allows for a greater prediction in estrogen deficiency than a measure of circulating estrogen of gonadal origin (31, 32). The expression of Cyp19 has been shown to be regulated by differential promoter usage, de-

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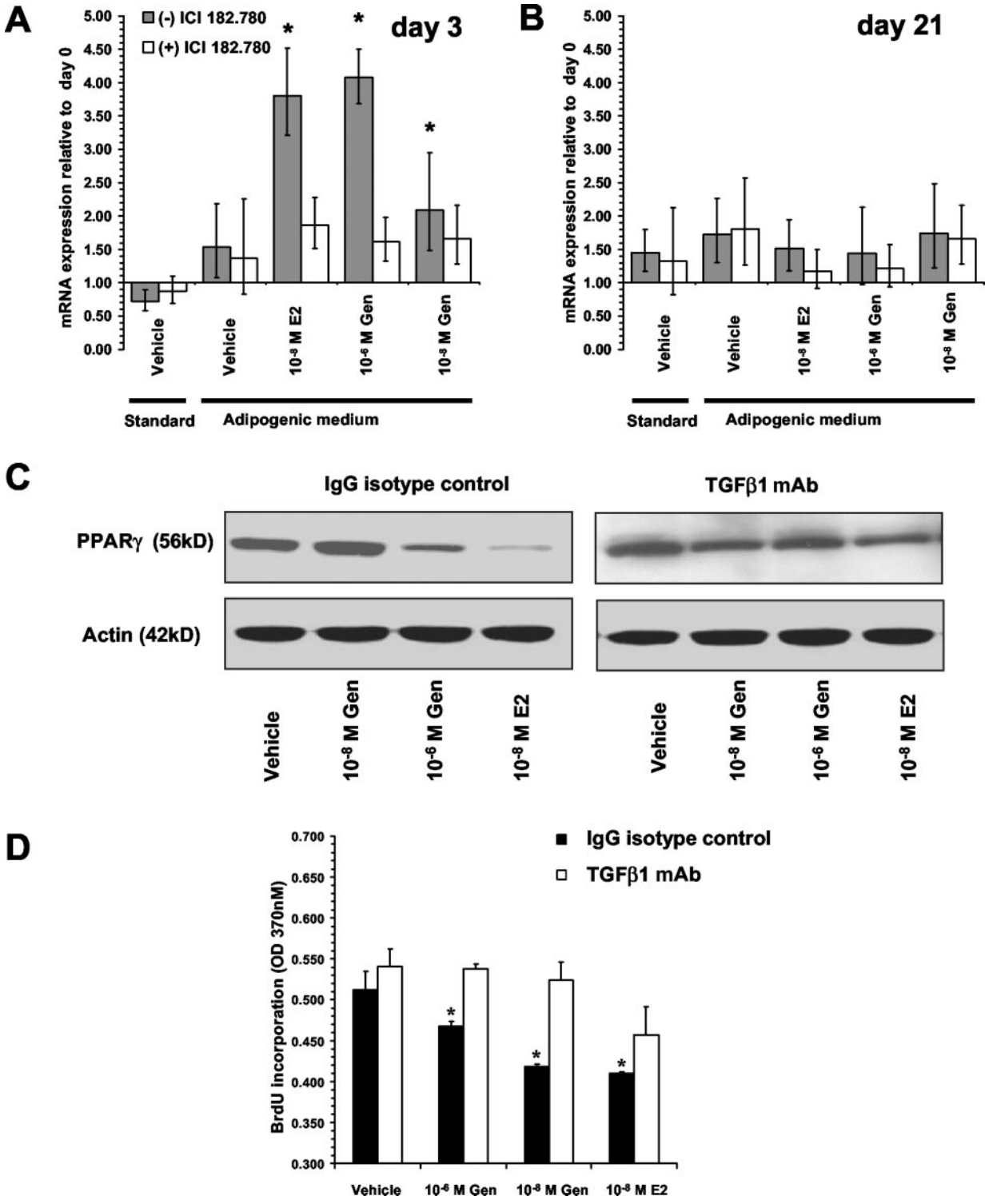

FIG. 6. TGFβ1-mediated repression of adipogenic differentiation and stromal cell/preadipocyte proliferation. TGFβ1 mRNA expression was determined at d 3 and 21 of adipogenic differentiation (A and B, respectively). BMSCs were grown in the absence (*white bars*) or presence (*gray bars*) of ICI 182.780 in DMEM, 1% FCS, 10^{-8} M insulin, and 10^{-7} M dexamethasone (AM) or in DMEM, 10% FCS (standard medium). The cells were treated during adipogenic stimulation with vehicle, 10^{-8} M genistein (Gen), 10^{-6} M genistein, or 10^{-8} M E2. For comparison nondifferentiated BMSCs treated with vehicle are shown on the left. The mRNA expression values were normalized to d 0. The influence of TGFβ1 on the PPARγ protein levels was measured by Western blot analysis (C). The cultures were treated for 3 d of adipogenic stimulation with an isotype antibody control or a neutralizing anti-TGFβ1-mAb in combination with vehicle, 10^{-8} M genistein, 10^{-6} M genistein, or 10^{-8} M E2. Actin was measured for internal control. Suppression of TGFβ1 signaling reduced the proliferation rate during adipogenic differentiation (D). The proliferation rates of cells committed to the adipogenic lineage and pretreated with the TGFβ1-mAb or a isotype control were determined using a BrdU incorporation assay at d 3 in the absence or presence of 10^{-8} M genistein, 10^{-6} M genistein, and 10^{-8} M E2.

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pending on the tissue context. In osteoblasts and adipocytes, aromatase is activated via the I.4 promotor (33). We observed a marked increase in Cyp19 aromatase mRNA levels during osteoblastic differentiation, which is consistent with the reports of others who have found aromatase expression and activity in mature osteoblasts (34, 35). During adipogenic differentiation a sharp increase in Cyp19 expression at the early commitment stage was observed, but the expression dropped to nearly undetectable levels on final differentiation. These results agree with those that show white fat tissue as a major site of Cyp19 expression and activity in the body. The Cyp19 expression seems to be restricted to stromal preadipocytes and not to be present in mature adipocytes (36). Interestingly, ligands for PPARγ, and thus activators of adipogenic differentiation, have been reported to be potent inhibitors of aromatase expression (37). Overall, these results led us to conclude that stromal/preadipocytic cells and mature osteoblasts are the major sources for local estrogen production in bone and that estrogen production may cease due to an increase in the number of mature adipocytes.

Genistein treatment enhanced the up-regulation of Cbfa-1 and the down-regulation PPARγ in osteoblastogenesis but reduced the down-regulation of Cbfa-1 and up-regulation of PPARγ in adipogenesis. This inverse regulation agrees with data showing that PPARγ negatively regulates stromal cell plasticity by suppressing Cbfa-1 and osteoblast cell activity (38). BMSCs undergoing osteogenic commitment can be divided into three different stages: 1) proliferation and commitment (d 0–7), which is accompanied by an increase in cell number and the inverse regulation of Cbfa-1 and PPARγ; 2) the differentiation phase (d 7–11), which goes along with an up-regulation of ALP expression and collagen type I; and 3) the maturation stage (beginning at d 15), which is characterized by increasing levels of OP, BSP-2, and induction of mineralized nodule formation. Our data imply that genistein acts similarly to E2 during late differentiation by up-regulating ALP expression and activity but has little or no effect on late maturation markers like BSP or OP.

Interestingly, the strongest effects of genistein and E2 on osteogenic gene expression are seen on OPG. OPG is a secreted decoy receptor for RANKL, which is expressed by stromal/osteoblast cells (39). RANKL is essential in the maturation and activity of osteoclasts (40). Undifferentiated bone marrow stromal cells with a low OPG:RANKL ratio can initiate and support osteoclastogenesis, but after differentiation toward the mineralizing osteoblastic phenotype, they lose this ability (41). Therefore, changing the relative ratio of OPG:RANKL in the osteoblast lineage has a major impact on the relative balance between osteoblast and osteoclast numbers. Estrogen and genistein have recently been demonstrated to be up-regulators of OPG in human trabecular osteoblast cultures (42, 43). In support of these data, we showed a progressive up-regulation in the OPG:RANKL ratio during differentiation, which is further enhanced by genistein and E2.

Generally, the higher genistein dose (1 μM) more effectively induced the regulation of marker genes during osteogenic differentiation. These results are valuable for understanding the role of genistein on the maintenance of bone homeostasis. Appropriate levels of daily genistein intake via nutrition or additional supplementation might be helpful for the prevention and management of increased bone marrow adipogenesis as seen in postmenopausal osteoporosis.

Recently Dang *et al.* (44) reported genistein as a novel ligand for PPARγ. Genistein competed binding for [^{3}H]-labeled rosiglitazone to full-length PPARγ with an approximate IC_{50} of 20 μM giving rise to a calculated K_i (inhibition constant) of 5.7 μM. A concentration of 10 μM genistein was able to increase the number of adipocytes in murine KS483 cultures and 25 μM genistein reversed the effects on murine osteogenesis. Concentrations of less than 1 μM of genistein, however, paralleled our findings in human BMSCs. Although these observations using pharmacological genistein concentrations are scientifically interesting, they have little biological relevance because serum levels in this concentration cannot be reached through nutrition.

We observed the up-regulation of TGFβ1 during late osteogenesis and at early time points of adipogenic commitment. TGFβ1 is a pleiotropic growth factor involved in cell proliferation and differentiation with both stage and cell type specificity. Three different TGFβ isoforms (β1, β2, and β3) that share high structural homology but seem to have differing effects on bone have been described. TGFβ1 has been shown to be a strong inhibitor of adipogenesis in 3T3 fibroblasts (45). Several effects on osteoblast proliferation, differentiation, and extracellular matrix production have been reported, although the results are somewhat conflicting (46). The cessation of ovarian estrogen production, such as that seen after menopause, leads to reduced TGFβ production in rat bone and has been implicated in estrogen deficiency-associated bone loss (47). In addition, increased TGFβ production by osteoblasts after estrogen treatment has been shown to enhance osteoclast apoptosis, suggesting that TGFβ acts as a coupling mediator between osteoblasts and osteoclasts (48). Our data show a role for TGFβ1 as a signaling factor in the genistein-mediated suppression of adipogenic proliferation and in the commitment of BMSCs. Whether the effect is autocrine or paracrine in nature remains to be elucidated because BMSC cultures contain a heterogeneous population of cells, and not all of these cells can be committed to the adipogenic lineage. We were able to show that this genistein-mediated effect is restricted to the early adipogenic differentiation stages. This is in agreement with the observations of Harmon *et al.* (49), who reported an inhibitory effect of genistein on 3T3-L1 preadipocytes, which was restricted to the early differentiation phase. During osteogenesis we observed that TGFβ1 consistently increased, an effect that was further enhanced by genistein in the final stages of osteoblast maturation. In contrast, during adipogenesis, there was an increase of ER-mediated TGFβ1 expression at d 3, but no regulation was found in response to the estrogenic stimulus at d 21. The ER profile (Fig. 1A) at d 3 conferred a much higher shift in adipogenesis than in osteogenesis that might explain this finding. These results clearly reveal stage-dependent estrogen-driven TGFβ1 regulation.

TGFβ1 signaling is mediated via type I and II membrane receptors (50), and expression of these receptors has been shown to decrease during adipogenesis (51). TGFβ affects PPARγ and C/EBPα transcriptional repression, which can be

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mediated via Smad signaling on the intracellular level (52) or by alternative pathways including the activation of MAPK signaling. It has been reported that TGFβ1 can regulate PPARγ activity on target gene expression through an MAPK/ERK phosphorylation cascade (53). Moreover, blocking ERK signaling with the inhibitor PD098059 blocks osteogenic differentiation and favors adipogenic differentiation (54). Given that genistein can activate MAPK/ERK signaling via an autocrine or paracrine TGFβ loop, it becomes a possible regulator of PPARγ phosphorylation and activity. The inhibitory effects of E2 on adipogenesis were also shown to be partially regulated via this TGFβ pathway, but the fact that interruption of TGFβ1 signaling did not completely abolish the E2-derived decrease of adipogenic commitment leads to the conclusion that additional effectors might be involved. For instance, we can speculate that E2 can rapidly activate receptor tyrosine kinase-linked pathways (55), whereas genistein, a known receptor tyrosine kinase inhibitor at supraphysiological concentrations (56), does not.

In conclusion, we have demonstrated that the natural food-derived compound genistein inversely regulates osteogenic and adipogenic commitment of BMSCs. It mimics the overall effects of the hormone E2 in reducing the final adipocyte count in cultures committed to the adipogenic lineage via the autocrine/paracrine TGFβ1 pathway. During osteogenesis, however, only high physiological concentrations of genistein proved to be able to induce early differentiation markers, whereas late differentiation and maturation markers were unaffected. These results impact our understanding on how commitment to the human osteoblast lineage is influenced. However, additional studies will be needed to resolve the exact role of the soy phytoestrogen genistein in the *in vivo* regulation of human bone metabolism.

Acknowledgments

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