JOURNAL OF BONE AND MINERAL RESEARCH
Volume 13, Number 4, 1998
Blackwell Science, Inc.

Immortalization and Characterization of Bone Marrow Stromal Fibroblasts from a Patient with a Loss of Function Mutation in the Estrogen Receptor-α Gene

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ABSTRACT

A male patient with abnormal postpubertal bone elongation was shown earlier to have a mutation in both alleles of the estrogen receptor, resulting in a nonfunctional gene. Marrow stromal fibroblasts (MSFs) derived from this patient were called HERKOs (human estrogen receptor knock outs), and in order to obtain continuous HERKO cell lines, they were immortalized using a recombinant adenovirus-origin-minus SV40 virus. MSFs are unique cells because they support hematopoesis and contain a mixed population of precursor cells for bone, cartilage, and fat. Three established cell lines (HERKO2, HERKO4, and HERKO7) were characterized and compared with the heterogeneous population of nonimmortalized HERKOs for their osteogenic potential. We performed Northern analysis of matrix genes implicated in bone development and metabolism and an in vivo bone formation assay by transplanting the cells subcutaneously into immunodeficient mice. All three HERKO lines expressed high amounts of collagen 1A1, osteopontin, osteonectin, fibronectin, decorin, biglycan, and alkaline phosphatase. Except for osteopontin, expression of these genes was slightly lower compared with nonimmortalized HERKOs. In the in vivo bone formation assay, the heterogeneous population of nonimmortalized HERKOs formed bone with high efficiency, while the HERKO lines induced a high-density, bone-like matrix. Finally, all HERKO cell types secreted high levels of insulin-like growth factor I and interleukin-6 into the culture medium relative to cells of normal human subjects. In summary, these lines of HERKO cells retain several of the phenotypic traits of MSFs after immortalization, including matrix and cytokine production, and provide a valuable source of a unique human material for future studies involving estrogen action in bone and bone marrow metabolism. (J Bone Miner Res 1998;13:598–608)

INTRODUCTION

Estrogen is well known in females to be important for bone development, growth, and metabolism. The increase in estrogen observed during puberty in girls induces epiphyseal fusion and bone mineral accretion.[1,2] Thirty to 40% of bone mineral mass is accrued during the pubertal years and appears to be a critical determinant of subsequent risk for osteoporosis.[3] Similarly, decrease in estrogen in postmenopausal women is associated with loss of bone

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mineral mass and estrogen replacement therapy inhibits the rate of bone mineral loss.[1,4]

Though in general terms the role of estrogen seems clear in females, less clear is the physiological role in men. Interpretation of estrogen action in vivo in males is complicated by the presence of androgen that is variably subject to aromatization into estrogen.[5] However, relative maintenance of bone mineral mass in patients with androgen insensitivity either with gonads present or treated with estrogen after gonadectomy suggests an important role for estrogen.[6] Consistent with these observations, adult males have been described with P450 aromatase deficiency.[7] These individuals present low bone density, delayed bone maturation, and tall stature. In one recently described case, estrogen therapy improved bone mineral mass and induced growth plate fusion.[8] Finally, identification of an adult male patient with a loss of function mutation in the estrogen receptor (ER)-α gene further emphasizes the importance of the estrogen signal transduction pathway.[9] This man presented a markedly low bone mineral density (BMD) and epiphyseal maturation delay (bone age, 15 years) and tall stature. Serum androgen levels were normal but estrogen concentrations were significantly elevated. Density of the lumbar spine was 0.745 gm/cm^2 (3.1 SD below the mean for a 15-year-old boy). Bone biopsy of the left iliac crest revealed a thin cortex and tapered but intact trabeculae.[10] Six months of transdermal estradiol therapy failed to either mature the epiphyses or improve BMD. DNA sequence analysis of the ER-α gene showed a mutation of a single base pair (bp) in the second exon in both alleles, generating a premature stop codon, theoretically resulting in a highly truncated protein lacking the DNA and hormone binding domains of the ER-α.

These observations all imply that estrogen may have similar functions in skeletal tissue of both males and females. Yet men with osteoporosis and females with androgen resistance have different phenotypic pictures compared with pure hypogonadism, e.g., cortical thickness is more preserved, suggesting that there are targets for androgen that are unique and distinct from estrogen.[11] Furthermore, the recent discovery of a second member of the ER family, ER-β, raises questions as to the relative role of these two receptors in mediating the actions of estrogen on bone.[12]

Absence of ER-α and its ligand, estradiol, have long been thought to be lethal, but the discovery of the ER-α–deficient patient and the creation of a mouse null for the ER-α (ERKO)[13] suggest the contrary. The ERKO mouse provides a useful model in evaluating and understanding estrogen's role in bone metabolism particularly since ERKO male mice show a lower BMD consistent with the clinical findings of the ER-α mutant patient.[9] However, there are some important differences between the mouse and the human model. ERKO mice have shorter legs, an unaltered serum alkaline phosphatase (ALP) content, and higher serum androgen levels compared with their wild-type littermates,[14,15] whereas the male patient has a tall stature, elevated ALP serum levels, and unaltered androgen levels. The difference between mice and men may be caused by species differences. For example, mice never establish closure of growth plates. Therefore, estrogen may affect skeletal development by acting via different pathways in mice and men. Development of immortalized cells containing the human knock out model system for ER-α may allow further elucidation of the relative roles of estrogen and androgen and the contribution of ER-α and -β in humans.

Toward this goal, we established cell lines of marrow stromal fibroblasts (MSFs) derived from the ER-α–deficient patient and characterized their properties. The MSFs were called human estrogen receptor knock outs (HERKOs) and were immortalized by infection with a genetically engineered virus that contained origin-minus SV40.[16] MSFs are a unique population of cells because they support hematopoesis and contain a mixed population of precursor cells that have the capacity to differentiate into numerous mesenchymal cell types including bone, cartilage, and fat, even after extensive proliferation in vitro.[17,18] Thus, by developing HERKOs into a continuous cellular model, the role of estrogen during growth and differentiation may be explored in a broad array of cell lineages. We established eight cell lines, and three of them (HERKO2, HERKO4, and HERKO7) were characterized and compared with HERKOs, the nonimmortalized source material. To drive the cells into the osteoblastic lineage, they were continuously treated with the glucocorticoid dexamethasone, which has previously been implicated to establish an osteoblastic differentiation status.[19] We examined the osteogenic potential of the cells by Northern analysis of matrix genes implicated in bone development and metabolism. All three HERKO lines expressed high amounts of collagen 1A1 (COL1A1), osteopontin, osteonectin, fibronectin, decorin, biglycan, and ALP. Except for osteopontin, expression of these genes was slightly lower compared with nonimmortalized HERKOs. In addition, we studied the bone-forming capacity of the cells in an in vivo transplantation assay[20] in which cultured human MSFs and HERKO cells were transplanted subcutaneously into immunodeficient mice, followed by examination on the presence of bone particles after several weeks. The heterogeneous population of nonimmortalized HERKOs formed bone with high efficiency, while the HERKO lines induced a high density, bone-like matrix. Finally, we determined secretion of IGF-I and IL-6 in the culture medium. All HERKO cell types secreted high levels of these factors into the culture medium relative to cells of normal human subjects.

MATERIALS AND METHODS

Cell culture and development of cell lines

Cancellous bone with bone marrow was obtained from the distal femur of the ER-α–deficient patient at age 29. The osteotomy was performed as a part of a leg-straightening procedure to correct severe genu valgum. Informed consent was obtained, and the use of the surgical waste material was approved by the Institutional Review Board. Nonimmortalized HERKOs and HERKO cell lines (i.e., HERKO-2, HERKO-4, and HERKO-7) were derived from an osteotomy sample as follows. Fragments of cancellous bone with bone marrow were scraped with a steel blade into

ice-cold alpha modified essential medium (α-MEM). After vigorously pipetting, the cell suspension was passed several times through 16 and 20 gauge needles and filtered through a 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were plated into 75 cm^2 flasks at 9×10^6 nucleated cells per flask and cultured in culture medium (α-MEM with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 10^{-8} M dexamethasone, and 10^{-4} M ascorbic acid) with 20% fetal bovine serum. After 1 week, medium was replaced to remove nonadherent cells. The adherent cells (HERKOs), consisting of a mixed population of marrow stromal fibroblasts, were harvested and reseeded. For immortalization, the HERKOs (passage 2) were seeded at 1.3×10^6 cells in 25 cm^2 flasks with culture medium with 10% fetal bovine serum. HERKOs were infected with an adenovirus-origin-minus wild type (wt) SV40 at a multiplicity of infection (MOI) of 50,[5] and after 1 week the cells were trypsinized and serially diluted. Medium was changed three times a week. Within 2 weeks, a dense cell layer was established, and after 1.5 months, distinct colonies of cells became apparent that grew on top of the cell layer. Different colonies from the same source material were developed into separate cell lines by isolating them with brief treatment of trypsin, followed by transfer with 200 μl pipette tips into 6-well plates. For RNA extraction, cells were cultured for 5–25 days in 60- or 100-mm culture dishes.

To study differential morphology of the heterogeneous source material, nonimmortalized HERKOs were plated at low density in 150 mm tissue culture dishes at 1×10^5 cells per dish to form single-colony derived strains.

Analysis of ER-α mutation on genomic DNA

Transformed cells from the ER-α–deficient patient and MSFs from control human subjects were cultured in 100 mm culture dishes until confluency, and genomic DNA was isolated by digesting the cultures overnight at 37°C with 4 ml Tris-EDTA (TE) containing 0.1 M NaCl, 1% sodium dodecyl sulfate (SDS), and 0.5 mg/ml proteinase K. Digests were extracted with buffered phenol and precipitated with 1 ml of ethanol:3 M NaOAc (25:1) for 1 h on dry ice. Precipitations were spun down, washed with 70% ethanol, lyophylized, and dissolved in 30 μl of TE.

The coding region of exon 2 of the ER-α gene was amplified by polymerase chain reaction (PCR) with an expected product of 216 bp. Primer sequences for exon 2 were synthesized considering previously described information on the exon–intron junction.[9] The PCR products were directly sequenced by an automated sequencer (ABI 370 A) using Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Branchburg, NJ, U.S.A.).

Growth curve analysis

Cells were seeded (40,000 cells/well) in triplicate for each time point in 24-well tissue culture plates. Cell growth curves were determined using a crystal violet staining technique.[21] The growth curves were modeled by fitting a Zweitering modified logistic.[21] The logistic fit was calculated using nonlinear regression with a Marquardt algorithm (DeltaGraph, DeltaPoint, Monterey, CA, U.S.A.). Statistical differences between the different cell types were determined by comparing distributions using the alternate Welch's *t*-test, which does not assume equal variances.

Isolation and analysis of expressed mRNAs

Total RNA was isolated using RNA STAT-60 (Tel-Test "B", Inc., Friendswood, TX, U.S.A.) according to manufacturer's instructions. For Northern analysis, 5–10 μg of total RNA was separated by electrophoresis in a 1.2% denaturing agarose gel.[22] RNA was transferred to a Zeta-Probe GT Blotting Membrane (BioRad, Hercules, CA, U.S.A.) by a capillary method using 10× SSC.[22]

A 537 bp cDNA probe for the large tumor (T) antigen was made by PCR using DNA from an adenovirus-origin-minus SV40 mutant (tsA209) as a template, and primer sequences located at nucleotide positions (gene accession #J02400) 2869–2891 5′-CCACCATCTTCATTTTTAT CA GC-3′ (sense) and 3383–3403 5′-TTGAGGATGTA AAG GGCACTG-3′ (antisense). cDNA probes for human COL1A1 (α1(I)-collagen), ALP, osteonectin, decorin, biglycan, osteopontin, and bone sialoprotein were generated as described previously.[23] RNA from cultured normal human trabecular bone was used as a positive control for bone sialoprotein.[24] A 330 bp cDNA encoding bovine osteocalcin was amplified by PCR from clone P426, a kind gift from Dr. L. Cooper (University of North Carolina). The osteocalcin cDNA was amplified by a PCR reaction using SP6 and T7 Promoter Primers (Invitrogen, San Diego, CA, U.S.A.). A human fibronectin cDNA was a kind gift from Dr. S. Aoto (National Institutes of Health, Bethesda, MD, U.S.A.). A 1.35 kb fragment of fibronectin cDNA was liberated from plasmid pRSETc by restriction digestion using *Xba*I and *Hind*III.[25] A 1050 bp *Eco*RI insert for TGF-β1 [26] was a gift from Dr. R. Derynck.

Approximately 100 ng of each cDNA probe was labeled using [α-^{32}P]dCTP (specific activity 3000 Ci/mmol) and Prime-It II Labelling Kit (Stratagene, La Jolla, CA, U.S.A.) following a protocol recommended by the manufacturer. Unincorporated radioactive [α-^{32}P]dCTP was removed with a G50 spin column (5Prime-3Prime, Inc., Boulder, CO, U.S.A.) or with a NucTrap purification column (Stratagene). Northern blots were prehybridized at 37°C in Hybrisol I (Oncor, Gaithersburg, MD, U.S.A.). Labeled cDNA probe was added directly into the prehybridization solution (10^7 cpm/membrane). The blot was washed in 2× SSC and 0.1% SDS at room temperature, subsequently washed in 0.1× SSC and 0.1% SDS at 65°C, and exposed to X-Omat film (Kodak, Rochester, NY, U.S.A.) for 2 h to 7 days at –70°C. To quantitate levels of mRNA, the bands in membranes or autoradiograms were scanned by PhosphorImager or Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.), respectively.

Reverse transcriptase (RT)-PCR for COL2A1 (collagen type II) was performed on RNA using primer sequences located at nucleotide positions (gene accession #X16468) 25–49 5′-TCGCTGGTGCTGCTGACGCTGCTCG-3′ (sense) and 206–228 5′-GGCACCTTTTTCACCTTTGT CAC-3′ (anti-

sense). RNA from human fetal limb used as a positive control was a kind gift from Dr. F. Luyten (National Institutes of Health, Bethesda, MD, U.S.A.). The PCR products were electrophoresed in 6% polyacrylamide TBE Gels (Novex, San Diego, CA, U.S.A.).

ALP and mineralization assays

Cells were cultured in 3-well SuperCell Culture Slides (VWR Scientific, Baltimore, MD, U.S.A.) and fixed and stained with an Alkaline Phosphatase kit (Sigma, St. Louis, MO, U.S.A.).

For mineralization, cells were cultured 16–25 days in 6-well plates in culture medium with or without 10 mM β-glycerophosphate. Medium was changed every 3 days. Postconfluent cultures were fixed with 4% paraformaldehyde and stained for calcium with alizarin red S and for calcium phosphate with von Kossa stain.

In vivo bone formation assay

In vivo transplantation of the cells was done as described previously.[20] Briefly, 1.8–3.0 × 10^6 cells (HERKOs, passage 4; HERKO2, 4; 7, 10–17) were loaded into a vehicle—40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer, Warsaw, IN, U.S.A.). To organize HA/TCP particles with cells attached to them, a secondary vehicle embracing the particles and consisting of fibrin clots was used in some experiments. Fibrin clots were prepared from mouse fibrinogen (3.3 mg/ml in 1× phosphate-buffered saline [PBS]) and mouse thrombin (25 U/ml in 2% $CaCl_2$; Sigma) by mixing 15 μl of each solution per transplant. Immunodeficient 8- to 15-week-old female beige mice (NIH-bg-nu-xidBR, Harlan Sprague-Dawley, Indianapolis, IN, U.S.A.) were used as subcutaneous transplant recipients, and up to four transplants per animal were placed on the dorsal surface. After 6 weeks, transplants were recovered and fixed for 5 h in 4% paraformaldehyde in PBS, subsequently decalcified for 9 days, in 10% EDTA for 10 days, fixed for another 2 h, and embedded in paraffin. After sectioning, sections were examined by hematoxylin-eosin staining and by in situ hybridization for human *alu* repetitive DNA sequences.

In situ hybridization for human alu

Digoxigenin-labeled probe specific for human *alu* sequences was prepared by PCR as described previously, and in situ hybridization was done as described with some modifications.[27] Briefly, deparaffinized sections were immersed in 0.2 N HCl at room temperature for 7 minutes, incubated in 1 mg/ml pepsin in 0.01 N HCl at 37°C for 10 minutes, washed in PBS, and washed in PBS with 50 mM $MgCl_2$ followed by a postfixation in 1% formaldehyde in PBS with 50 mM $MgCl_2$. After washing in PBS, the sections were treated with 0.25% acetic acid containing 0.1 M triethanolamine (pH 8.0) for 10 minutes, washed in PBS, dehydrated in ethanol, and prehybridized with 70% deionized formamide in 2× SSC at 80°C for 3 minutes The sections were then quenched on ice cold 70% ethanol, dehydrated in ethanol, and hybridized with 1 ng/μl probe in hybridization buffer[27] at 42°C for 16 h. Digoxigenin-labeled DNA was detected by immunohistochemistry using antidigoxigenin ALP-conjugated Fab fragments (Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.).

IGF-I and IL-6 immunoassays

IGF-I levels were determined in medium samples after an acid-ethanol extraction followed by radioimmunoassay using a kit purchased from Nichol Diagnostics Inc. (San Juan Capistrano, CA, U.S.A.). The radioimmunoassay was developed following the manufacturer's protocol, except that a 1:15 dilution on the medium was used. In our laboratory, this assay has an interassay percentage coefficient of variance (% CV) of 9.26. IL-6 levels were determined using an enzyme-linked immunosorbent assay (ELISA) obtained from Endogen Inc. (Cambridge, MA, U.S.A.) following manufacturer's protocol. This assay has an interassay percentage CV of 8.87. Samples were analyzed in duplicate for both assays, and mean values were corrected for cell number at each time point (every other day). Values were averaged across the entire growth curve and multiple growth curves to yield a mean value of steady-state cytokine production. Statistical differences were compared using an alternate Welch's *t*-test, which does not assume equal variances.

RESULTS

T antigen–expressing cell lines

Eight cell lines of HERKO MSFs were established and three of them (HERKO2, HERKO4, and HERKO7) were characterized and compared with the original, nonimmortalized HERKOs. To confirm that the immortalized cells contained the ER-α mutation, the coding region of exon 2 of the ER-α gene was amplified by PCR and compared with control subjects. Sequencing of the PCR product of the immortalized HERKO cells showed a single base pair (bp) substitution at codon 157, resulting in the replacement of an arginine codon (CGA) with a theoretical premature stop codon (TGA). Human MSFs from control subjects displayed a wild-type sequence. Furthermore, Western analysis using antibody ER21 (a kind gift from Dr. G. Green, University of Chicago) showed that ER-α was undetectable in the HERKO cells while present in control MSFs (data not shown).

To determine whether infection with adenovirus-origin-minus SV40 was successful, we constructed a cDNA probe by PCR for the large T antigen with a product of 537 bp (see Materials and Methods). Sequencing of the PCR product confirmed the expected T-antigen coding region. Hybridization of mRNA from cultured cells showed that the SV40-infected cell lines all expressed the large T antigen with high abundancy but cultured human MSFs from control subjects did not (Fig. 1A).

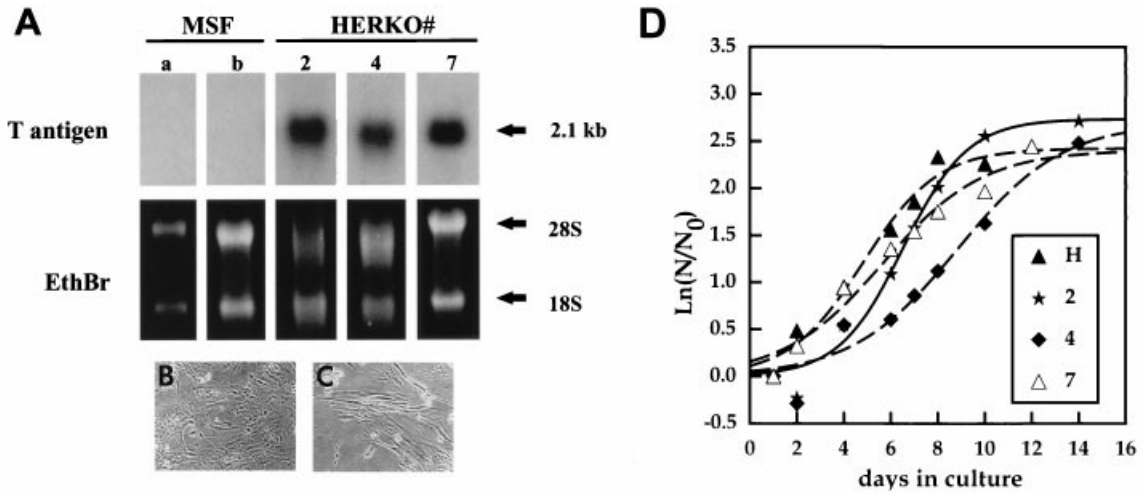

FIG. 1. (A) mRNA expression of large T antigen in different cell types. Lanes a and b represent nonimmortalized MSFs from two normal patients. Established cell lines HERKO2, HERKO4, and HERKO7 are positive for T antigen. Ethidium bromide staining of ribosomal RNA 28S and 18S in the same gel is shown in the bottom panel. Oligo, oligonucleotide primer; M, markers; bp, basepairs; kb, kilo basepairs. (B,C) Photographs of cultures of (B) HERKO2 and (C) HERKO7. Original magnification ×100. (D) Representative growth curves of HERKO (O), HERKO2 (2), HERKO4 (4), and HERKO7 (7). Cells were plated at 40,000 cells/well and fixed in 1% glutaraldehyde at days indicated. Cultures were stained for crystal violet and processed to profile growth curves. The maximal growth rate of HERKO2 was significantly different from others ($p < 0.05$). N, absorbance at day indicated; N0, initial absorbance value.

Morphology and growth

Plating of nonimmortalized HERKOs at very low cell density formed discrete single-colony derived strains of cells which showed a differential morphology varying in shape from spindle-like through "intermediate" to flattened cells. The majority, five out of eight strains, displayed a spindle shape. Multicolony-derived HERKOs appeared spindle-shaped upon reaching confluency. Subconfluent and confluent cultures of the SV40-infected HERKO cell lines appeared less spindle-shaped, with HERKO2 having a strikingly ellipsoid appearance compared with the others (Fig. 1B). Both HERKO4 and HERKO7 cells (Fig. 1C) showed an "intermediate" shape and produced a substantial amount of cell particulate matter. HERKO2 showed the highest maximal growth rate (Fig. 1D) and reached the highest passage number (passage 22) upon continuous passage, at which time the cells stopped growing well. Trypsinization of the different cell lines also showed major differences. HERKO2 could be easily removed from the culture plate as one sheet of cells, while HERKO4 was difficult to detach from the culture dish; HERKO7 was released as clumps of cells.

mRNA expression

The expression of several collagen and noncollagenous genes was studied to determine whether the cells retained their MSF phenotypic character after infection with adenovirus-origin-minus wt SV40. Total RNA was recovered from 5–20 day cell cultures in passage 4 and 8 for nonimmortalized cells and passage 8–12 for immortalized cells. Figure 2 and Table 1 are summarized data from two repeated experiments. The immortalized and nonimmortalized cell cultures showed similar expression patterns. All cell types were negative for bone sialoprotein, osteocalcin, and COL2A1. The proteoglycans, biglycan and decorin, were highly abundant, as were the noncollagenous proteins osteopontin, osteonectin, and fibronectin. In addition, TGF-β1 was present in high amounts. Some of the genes were expressed at relatively different levels in the immortalized cell lines in comparison with the nonimmortalized cells (Table 1). Specifically, biglycan and decorin showed a lower abundancy in all HERKO cells, COL1A1 a lower abundancy for HERKO2 and HERKO7, and osteopontin was expressed at higher levels in HERKO4 and HERKO7 cells.

Expression of ALP and mineralization

The nonimmortalized HERKOs expressed large amounts of ALP message (Fig. 3A). The HERKO cell lines also had high ALP message expression, but the levels were relatively lower in comparison with the nonimmortalized cells. Because cultures stained positive for ALP, this indicated that the mRNA was translated into protein in all cell types (Fig. 3B and data not shown).

Long-term cultures with β-glycerophosphate showed that the nonimmortalized cells formed nodules followed by their mineralization. Staining with von Kossa and alizarine red S showed the presence, respectively, of calcium and calcium phosphate in the nodules (Figs. 4A and 4B). In the immortalized cell lines, no nodules became apparent and only HERKO4 was positive for calcium and calcium phosphate, albeit precipitated throughout the culture dish (Fig. 4C). Without β-glycerophosphate, none of the cell types (nonimmortalized or immortalized) mineralized.

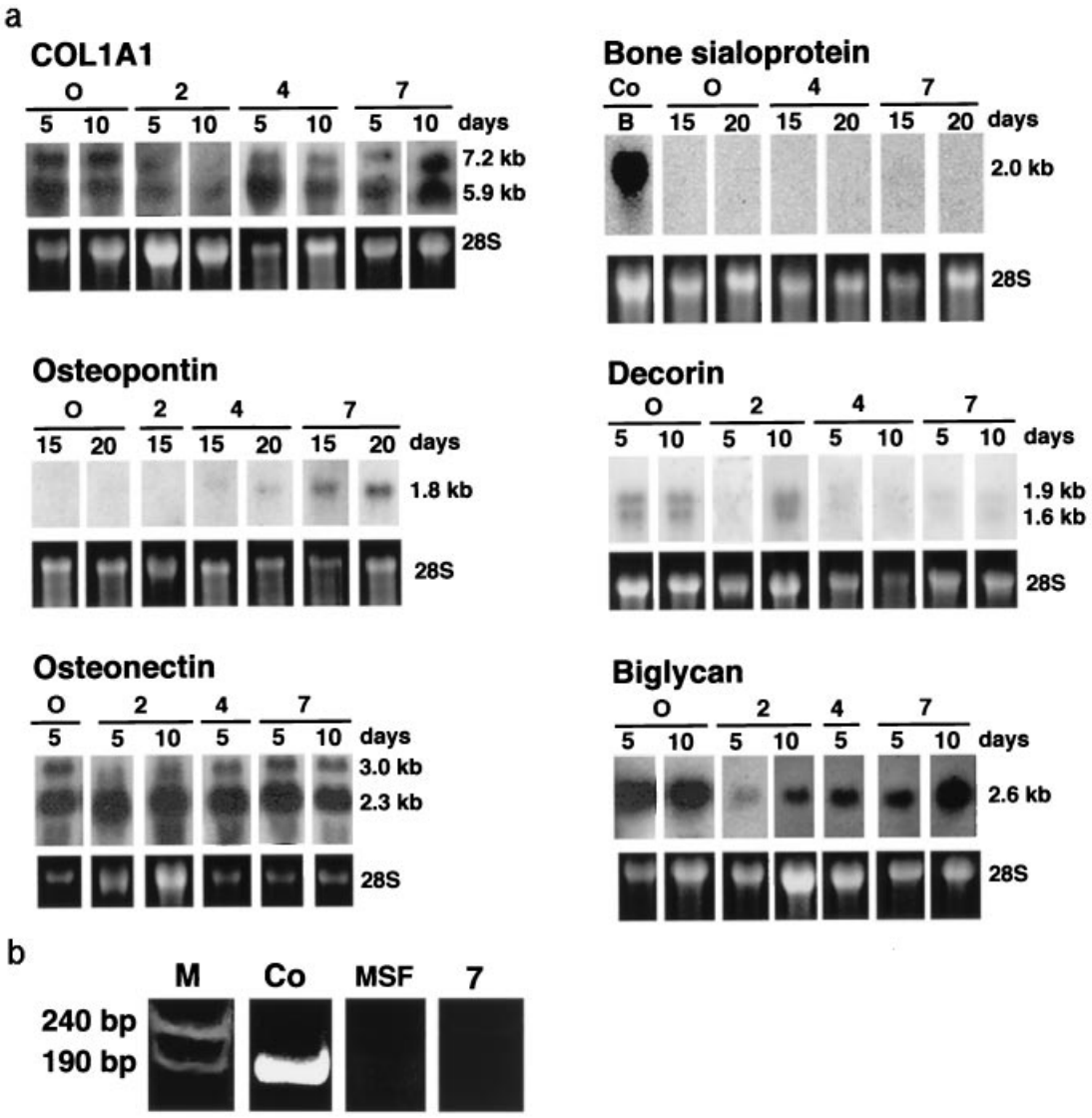

FIG. 2. (A) Upper panels, Northern analysis on COLI A1, osteopontin, osteonectin, bone sialoprotein, decorin, and biglycan of HERKO (O), HERKO2 (2), HERKO4 (4), and HERKO7 (7) on days indicated. Sizes of mRNA are denoted by kb (kilo basepairs). Lower panels, ethidiumbromide staining of ribosomal RNA 28S in the same gel. Co (control), RNA from cultured normal human trabecular bone (b). (B) RT-PCR for COL2A1 of mRNA from HERKO7 (7) and human MSFs. M, markers; Co (control), RNA from human fetal limb.

In vivo bone formation

Nonimmortalized HERKOs were cultured at passage 4, loaded into an HA vehicle, and transplanted subcutaneously into immunodeficient mice. After 6 weeks, new bone was deposited against the vehicle surface with high efficiency (Fig. 5A). Areas of active hematopoesis surrounded the bone particles, attributing to a new bone marrow organ. The bone exhibited lacunae-containing osteocytes as well as osteoblastic layers on both the inner and outer surfaces. In situ hybridization was performed with a probe for human *alu,* a human-specific repetitive sequence which comprises about 5% of the total human genome. This was used to show that these cells were of human origin (Fig. 5B). In some areas fibroblastic human *alu*-positive cells were found in the marrow (data not shown). In implants of the cell lines, some bone was visible, but for the most part a bone-like high-density matrix was induced by all cell types (Fig. 5C). Only a few human *alu*-positive cells were present (data not shown).

IGF-I and IL-6 production

Steady-state levels of IGF-I and IL-6 were determined by analyzing culture medium derived from the growth curve studies by immunoassays specific for each factor. Normal human MSFs produced 43 $\pm$ 6 ng of IGF-I/105 cells and

TABLE 1. SUMMARY OF RELATIVE mRNA EXPRESSION

Gene	*HERKO*	*HERKO2*	*HERKO4*	*HERKO7*
COL1A1	+++	+	+++	++
COL2A1	−	−	−	−
Osteopontin	+	+	++	+++
Osteonectin	+++	+++	+++	+++
Osteocalcin	−	−	−	−
Bone sialoprotein	−	−	−	−
Fibronectin	+	+	+	+
Decorin	+++	++	+	+
Biglycan	+++	+	++	++
TGF-β1	++	++	++	++
Alkaline phosphatase	+++	++	++	++

331 ± 46 pg of IL-6/105 cells on day 2 of culture. For comparison, the levels of these cytokines produced by human osteoblast-like cells derived from a normal 27-year-old male were determined on day 2 of culture and found to be 38 ± 6 ng of IGF-I/105 cells and 66 ± 10 pg of IL-6/105 cells. In contrast, HERKO MSFs produced 90 ± 18 ng of IGF-I/105 cells and 851 ± 160 pg of IL-6/105 cells on the second day of culture. The steady-state levels produced by the three immortalized HERKO cell lines were also approximately 2-fold higher than normal human MSFs, ranging from 99 ± 9 to 131 ± 5 ng of IGF-I/105 cells and from 598 ± 90 to 896 ± 116 pg of IL-6/105 cells on day 2.

The values for steady-state levels of IGF-I and IL-6 on days 2, 4, 6, 8, and 10 were averaged between replicate growth curves for each cell type, and the net steady-state levels were calculated by combining and averaging all time points. When the net steady-state levels of IGF-I and IL-6 were compared, the following differences were observed (Fig. 6). In comparison to IGF-I values from normal human MSFs, the average net production of IGF-I was significantly elevated in the HERKOs and immortalized HERKO2, HERKO4, and HERKO7 cells ($p < 0.001$, $p < 0.01$, $p < 0.005$, and $p < 0.05$, respectively). Between the nonimmortalized and immortalized HERKO cells there was no significant difference in IGF-I production. Similarly to IGF-I, net steady-state levels of IL-6 were significantly different between normal human MSFs and HERKO cells ($p < 0.005$), while there was no significant difference between the nonimmortalized HERKO cells or any of the immortalized cell lines (HERKO2, HERKO4, and HERKO7).

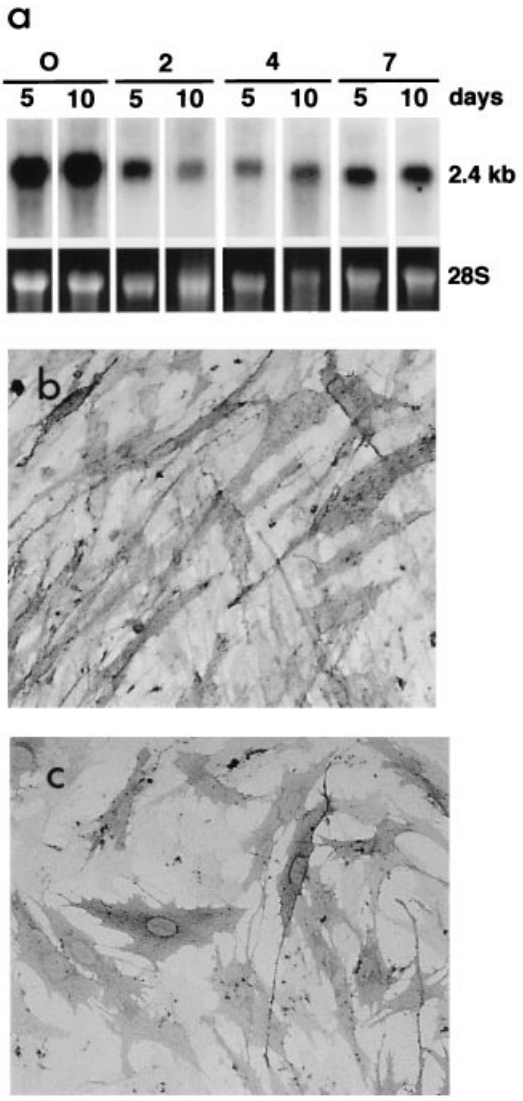

FIG. 3. (a) Upper panel, mRNA expression of alkaline phosphatase by nonimmortalized HERKOs (O), HERKO2 (2), HERKO4 (4), and HERKO7 (7) on days 5 and 10 as indicated. Lower panel, ethidium bromide staining of ribosomal RNA 28S in the same gel. (b,c) ALP staining on HERKO (b) and HERKO4 (c).

DISCUSSION

In this study, we present the characterization of three cell lines established from a heterogeneous population of human MSFs derived from an ER-α–deficient patient. DNA analysis confirmed that the cells contain the ER-α mutation and further have undetectable protein, suggesting that the ER-α is not functional. When the cells were treated with estrogen, no difference in mRNA expression patterns were observed using numerous sets of oligoprimers in extensive differential display analysis (L. Fisher, personal communication). Taken together, we speculate that the cells have little or no response to estrogen presumably due to the lack of ER-α protein expression.

Multicolony-derived strains of human MSFs can differentiate into various mesenchymal lineages depending on culture conditions. We attempted to drive the nonimmortalized HERKOs and the HERKO cell lines into the osteoblastic lineage by treating them continuously with the glucocorticoid dexamethasone. Therefore, we focused primarily on biochemical markers such as expression of matrix proteins, believed to represent early and late phases of osteoblast differentiation. During the various stages of the osteoblastic lineage, these matrix proteins are present

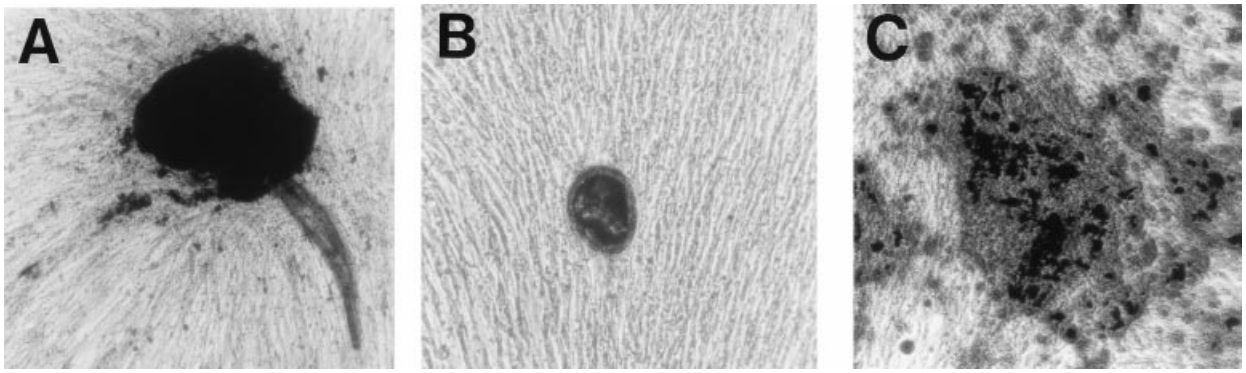

FIG. 4. Nonimmortalized HERKOs stained with (A) von Kossa or (B) alizarine red S and (C) HERKO4 stained with both von Kossa (black areas) and alizarine red (gray areas) after long-term culture with β–glycerophosphate. (A,B) Nonimmortalized HERKOs formed mineralized nodules. (C) HERKO4 displayed a precipitation of mineral throughout the culture dish.

as particular subsets, depending on cell type, cell age, and cell culture system. In MSF cultures of the original source material (HERKOs), we found high expression levels of the broadly expressed genes COL1A1, decorin, osteonectin, biglycan, and ALP (both RNA and protein), low levels of osteopontin and fibronectin, and no detectable levels of the late phase markers bone sialoprotein and osteocalcin. The established immortalized cell lines showed similar expression patterns for these genes, suggesting that they retain several of their phenotypic traits after immortalization.

The three cell lines clearly have different phenotypes as seen by their growth and morphology and surface attachment characteristics. In addition, they show subtle differences in the levels of expression in several genes (COL1A1, osteopontin, decorin, and biglycan), which might be related to their different phenotype. Several of the matrix genes studied here are thought to play a role in mineralization of bone matrix, the final stage of bone development. Osteonectin (SPARC, BM-40) is a glycoprotein widely expressed by osteoblasts and periosteal cells, and to a lesser extent osteocytes and hypertrophic chondrocytes.[28,29] In the HERKO cells and HERKO cell lines, osteonectin expression was high, thereby potentially establishing a suitable environment for mineralization. However, mineralization did not occur in all cell types. The nonimmortalized HERKO cells exhibited formation of nodules followed by their mineralization, as has been described in cultures of normal MSFs,[30] but the HERKO cell lines did not. The inability of cell lines to establish prototypical mineralization might be related to the lower expression levels of ALP, an enzyme thought to play a crucial role in mineralization. Interestingly, among the different cell lines, HERKO4, the only one that displayed some degree of mineralization, also exhibited the highest expression levels of COL1A1. This predominant collagen in bone is thought to provide a scaffold to organize the proteins that mediate mineral deposition in the form of hydroxyapatite.[31,32] Osteopontin and several other matrix proteins have been shown to contain Arg-Gly-Asp (RGD) sites, which are thought to bind cell surface receptors, thereby mediating cell attachment (reviewed by Robey and Boskey 1996).[31] The differences in matrix gene expression in these cell lines may ensue differences in cell–matrix adhesion and in turn cell surface attachment. Since the cell lines were established from a heterogeneous cell population, they may represent subpopulations of the original nonimmortalized cells, thereby expressing different phenotypes. However, we cannot rule out the possibility that the SV40 gene may be incorporated in a differential fashion in the genome among the different cell lines or that the SV40 incorporation causes the cell to revert into a fetal phenotype, as suggested in studies using keratinocytes.[33]

Consistent with the capacity to form mineralized nodules, the nonimmortalized HERKOs were able to form bone in vivo. The immortalized cell lines did not form substantial amounts of bone, but induced a high-density bone-like matrix. Recently, Kuznetsov et al.[27] showed that when MSFs are fractionated into single colony–derived strains, 59% of the strains form bone. It is possible, therefore, that the cell lines we selected from the heterogeneous source material may represent a cell type not able to form bone in this assay. It is also possible that the heterogeneous population of nonimmortalized HERKOs might have the advantage of different cell types cooperating together to form bone. Thus, the cell lines represent a single cell and subsequently might lack the supporting environment from other cell types which may be required for osteogenesis. Since human *alu*-positive cells were virtually absent in the implants of the cell lines, we assume the cells did not survive or alternatively migrated out of the implant.

Several factors implicated in playing an intermediary role for estrogen regulation of bone metabolism were also examined. Such factors are thought to control the subtle balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation via paracrine or autocrine pathways, and various factors have been nominated as local mediators. In cultures of human osteoblast-like cells, estrogen increases the synthesis and secretion of TGF-α,[34] and in avian osteoclasts a direct action of estrogen to lower resorption activity may be mediated by autocrine/paracrine production and activation of TGF-α.[35] IL-6 has been reported to be involved in the process of the stimulated

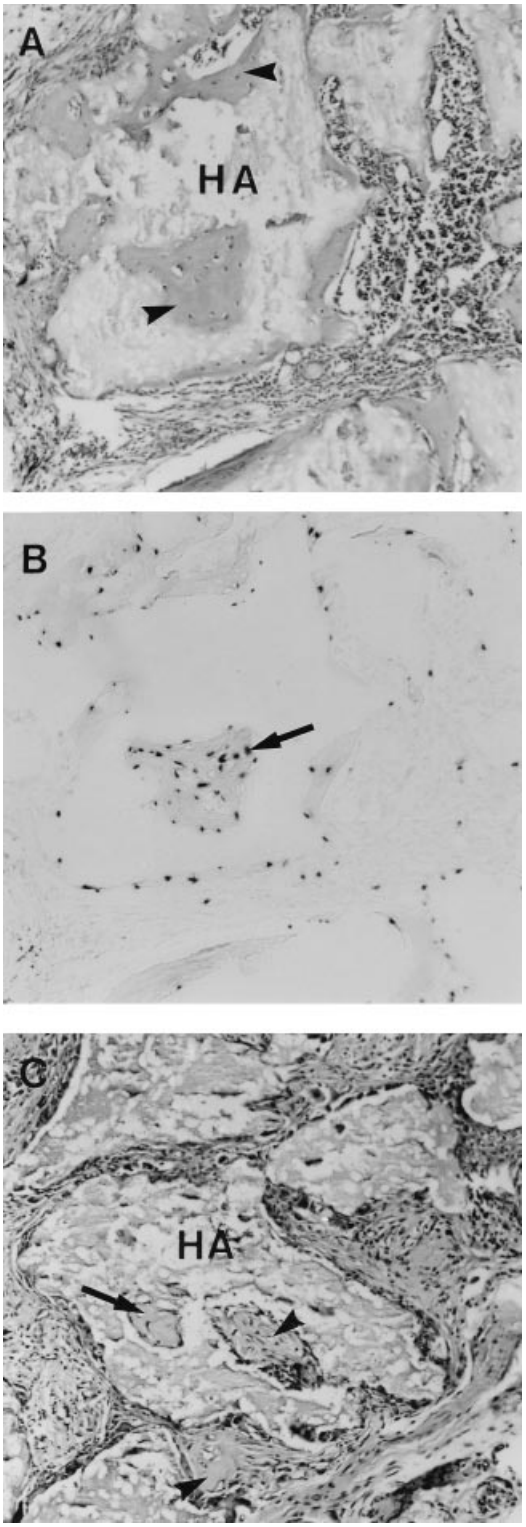

FIG. 5. (A,B) Bone formation by nonimmortalized HERKOs in vivo. Six-week transplants were analyzed by (A) HE staining and (B) in situ hybridization for human *alu* on the same implant. Note human *alu*-positive cells (arrow) present within the bone particles (arrowheads). HA, remnant of HA/TCP powder. (C) Formation of a small bone particle (arrow) and a bone-like high density matrix (arrowheads) induced by HERKO4 cells. Original magnification ×100.

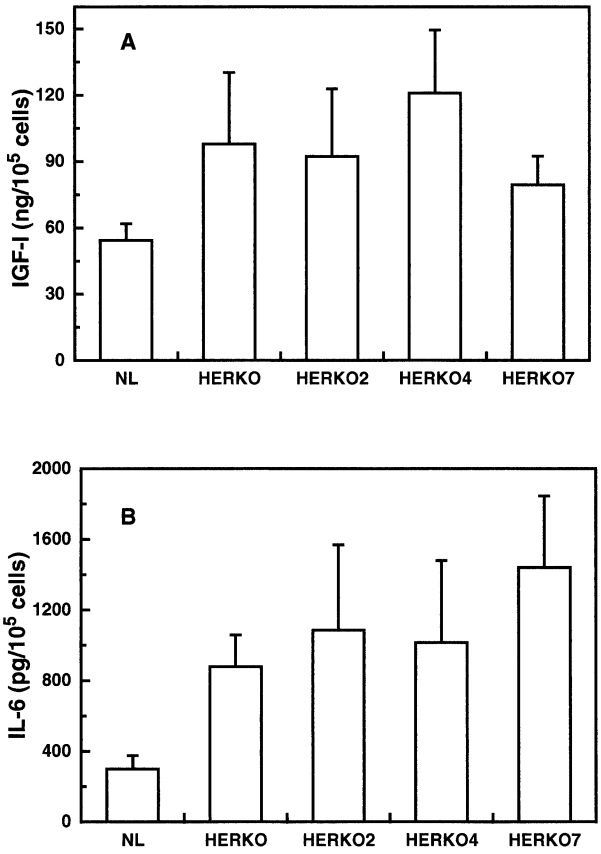

FIG. 6. Average production of (A) IGF-1 and (B) IL-6 into the culture medium by MSFs from a normal patient (NL), and HERKO, HERKO2, HERKO4, and HERKO7. The production by NL was significantly different from others ($p < 0.01$).

osteoclastic bone resorption induced by estrogen deficiency.[36] Strikingly, ovariectomy of IL-6-deficient mice does not induce any change in either bone mass or bone remodeling.[37] In ovariectomized rats, the increase in serum levels of IGF-I could be inhibited by estrogen supplementation.[38,39] All cell types studied here expressed TGF-β1 mRNA and produced high levels of IL-6 and IGF-I. Interestingly, production of IGF-I in the medium was high compared with normal human MSFs and osteoblastic cells. This corresponds well with the elevated serum levels of IGF-I in the ER-α–deficient male and it may imply that osteoblastic cells originating from human MSFs are a major source of circulating IGF-I in the ER-α–deficient male. However, the IGF-I concentrations in the patient were normal for a midpubertal boy, likely reflecting the ongoing active linear growth in this individual. Suppression of IGF-I and IL-6 production, by introducing functional ER-α in the HERKO cells in the presence of estrogen, might give clues to its interaction with these factors, and preliminary data from our lab indicate that estrogen may inhibit IL-6 secretion in ER-α–transfected HERKO cells in the presence of TGFβ-1.

It has been shown that ER-α is able to work in the absence of ligand, via the Ras-MAPK cascade of the growth

factor signaling pathways using the AF-1 position of the ER-α.[40] The AF-1 covers the N terminus of the gene in a region upstream from the mutation. The cells from the ER-α–deficient male described in this study have a disruption of the gene in exon 2, where, theoretically, the AF-1 domain would be unaffected. Since the ER-α–deficient male had apparently normal development until puberty, it is tempting to suggest that ER-α in males might function via the Ras-MAPK cascade until puberty, but need the estrogen ER-α–mediated signaling pathway for epiphyseal closure and maintenance of bone metabolism. Alternatively, estrogen might work via other pathways such as the protein kinase C signal transduction pathway.[41] This pathway or alternate regulatory pathways may be abundant and subsequently dominate or supperride the classical estrogen–ER interaction. In this context, it is important to note that recently a novel receptor for estrogen (ER-β) was discovered in humans,[42] and it has been suggested that ER-α and ER-β play different roles in gene regulation.[43] Using RT-PCR, we found that the HERKO cells express ER-β (data not shown), and since the HERKO cells are ER-α–deficient, they provide a potential model to study ER-β's functions.

In summary, we established several lines of human ER-α–deficient cells that, judged by their cell morphology, growth, and matrix production, retain several of their phenotypic traits after immortalization. These cell lines will provide a valuable source of a unique human material for future studies involving estrogen action in bone metabolism by clarifying the relative roles of androgens and estrogens and the contribution of ER-α and ER-β.

ACKNOWLEDGMENTS

The authors thank Dr. Bert Bachrach for transporting the bone sample of the patient to our laboratory at the National Institutes of Health. Dr. N.S. Fedarko was supported by NIAMS grant R01 AR42358.

REFERENCES

1. Turner RT, Riggs BL, Spelsberg TC 1994 Skeletal effects of estrogen. Endocr Rev **15:**275–300.
2. Cutler GB Jr, Cassorla FG, Ross JL, Pescovitz OH, Barnes KM, Comite F, Feuillan PP, Laue L, Foster CM, Kenigsberg D, Caruso-Nicoletti M, Garcia HB, Uriarte M, Hench KD, Skerda MC, Long LM, Loriaux DL 1986 Recent Prog Horm Res **42:**443–470.
3. Bachrach LK 1993 Bone mineralization in childhood and adolescence. Curr Opin Pediatr **5:**467–473.
4. Gambert SR, Schultz BM, Handy RC 1995 Osteoporosis: Clinical features, prevention and treatment. Endocrinol Metab Clin North Am **24:**317.
5. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR, Bulun SE 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev **15:**342–355.
6. Soule SG, Conway G, Prelevic GM, Prentice M, Ginsburg J, Jacobs HS 1995 Osteopenia as a feature of the androgen insensitivity syndrome. Clin Endocrinol **43:**671–675.
7. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. Clin Endocrinol Metab **80:**3689–3698.
8. Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med **337:**91–95.
9. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med **331:**1056–1061.
10. Bachrach BE, Beck AM, Li XJ, Specker B, Tsang RC, Kirk RM, Cohen RC, Williams TC, Korach KS, Smith EP 1995 Bone histomorphometric analysis of a 29 year old man with estrogen resistance caused by an estrogen receptor mutation: Severe osteopenia and evidence for decreased osteoblastic and osteoclastic activity. The Endocrine Society Meetings, Washington, DC, U.S.A., June 14–17.
11. Orwoll ES, Bliziotes M 1994 Heterogeneity in osteoporosis: Men versus women. Rheum Dis Clin North Am **20:**671–689.
12. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA **93:**5925–5930.
13. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA **90:**11162–11166.
14. Korach KS, Taki M, Kimbro KS 1997 The effects of estrogen receptor gene disruption on bone. In: Paoletti R (ed.) Proceedings of the 2nd International Symposium on Women's Health and Menopause: Risk Reduction Strategies. Kluwer Academic Publishers, Boston, MA, U.S.A., Vol. 11.
15. Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology **137:**4796–4805.
16. Lei K-J, Gluzamn Y, Pan C-J, Chou JY 1992 Immortalization of virus-free human placental cells that express tissue-specific functions. Mol Endocrinol **6:**703–712.
17. Friedenstein AAJ, Chailakhyan RK, Gerasimov UV 1987 Bone marrow osteogenic stem cells: In vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet **20:**263–272.
18. Owen ME 1988 Marrow stromal stem cells. J Cell Sci Suppl **10:**63–76.
19. Cheng S-L, Yang JW, Rifas L, Zhang S-F, Avioli LV 1994 Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. Endocrinology **134:**277–286.
20. Kresbach PH, Kuznetsov SA, Satomural K, Emmons RVB, Rowe DW, Gehron Robey M 1997 Bone formation in vivo: Comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation **63:**1059–1069.
21. Fedarko NS, D'Avis P, Frazier CR, Burrill MJ, Fergusson V, Tayback M, Sponseller PD, Shapiro JR 1995 Cell proliferation of human fibroblasts and osteoblasts in Osteogenesis Imperfecta: Influence of age. J Bone Miner Res **10:**1705–1712.
22. Sambrook J, Fritsch EF, Maniatis T 1989 Appendix E: Commonly used techniques in molecular cloning. In: Sambrook J Fritsch EF, Maniatis T (eds.) Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A., pp. E1-E39.
23. Ibaraki K, Termine JD, Whitson SW, Young MF 1992 Bone matrix mRNA expression in differentiating fetal bovine osteoblasts. J Bone Miner Res **7:**743–754.
24. Robey PG, Termine JD 1985 Human bone cells in vitro. Calcif Tissue Int **37:**453–460.
25. Danen EHJ, Aota S, van Kraats AA, Yamada KM, Ruiter DJ, Muijen GNP 1995 Requirement for the synergy site for cell adhesion to fibronectin depends on the activation state of integrin a5β1. J Biol Chem **270:**21612–21618.
26. Derynck R, Jarett JA, Chen EY, Eaton DH, Bell JR, Assoian

K, Roberts AB, Sporn MB, Goeddel DV 1985 Human transforming growth factor β complementary DNA sequence and expression in normal and transformed cells. Nature **316:**701–705.
27. Kuznetsov SA, Kresbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Gehron Robey P 1997 Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res **12:**1335–1347.
28. Holland PWH, Harper SJ, McVey JH 1987 In vivo expression of mRNA for the Ca++-binding protein SPARC (osteonectin) revealed by in situ hybridization. J Cell Biol **105:**473–482.
29. Metsaranta M, Young MF, Sandberg M, Termine J, Vuorio E 1989 Localization of osteonectin expression in human fetal skeletal tissues by in situ hybridization. Calcif Tissue Int **45:** 146–152.
30. Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I 1994 Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol **161:**218–228.
31. Robey PG, Boskey AL 1996 The biochemistry of bone. In: Marcus R, Feldman D, Bilizekian JP, Kelsey J (eds.) Osteoporosis. Academic Press, NY, U.S.A., pp. 95–183.
32. Glimcher M 1984 Recent studies of the mineral phase in bone and its possible linkage to the organic matrix by protein-bound phosphate bonds. Phil Trans R Soc London B **304:**479–508.
33. Bernard BA, Robinson SM, Semat A, Darmon M 1985 Reexpression of fetal characters in simian virus 40-transformed human keratinocytes. Cancer Res **45:**1707–1716.
34. Oursler MJ, Cortese C, Keeting P, Anderson MA, Bonde SK, Riggs BL, Spelsber TC 1991 Modulation of transforming growth factor-β production in normal human osteoblast-like cells by 17 β-estradiol and parathyroid hormone. Endocrinology **129:**3313–3320.
35. Robinson JA, Riggs BL, Spelsberg TC, Oursler MJ 1996 Osteoclasts and transforming growth factor-β: Estrogen-mediated isoform-specific regulation of production. Endocrinology **137:** 615–621.
36. Manolagas SC, Jilka RL, Girasole G, Passeri Bellido T 1993 Estrogen, cytokines, and the control of osteoclast formation and bone resorption in vitro and in vivo. Osteoporos Int **1** (Suppl)**:**S114–S116.
37. Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H, Ciliberto G, Rodan GA, Costantini F 1994 Interleukin-6 deficient mice are protected from bone loss caused by estogen depletion. EMBO J **13:**1189–1196.
38. Sato F, Ouchi Y, Masauyama A, Nakamura T, Hsoi T, Okamoto Y, Sasaki N, Shiraki M, Orimo H 1993 Effects of estrogen replacement on insulin-like growth factor I concentrations in serum and bone tissue and on interleukin 1 secretion from spleen macrophages in oophorectomized rats. Calcif Tissue Int **53:**111–116.
39. Kalu DN, Arjmandi BH, Liu C-C, Salih MA, Birnhaum RS 1994 Effects of ovariectomy and estrogen on the serum levels on insulin-like growth factor-I and insulin-like growth factor binding protein-3. Bone Miner **25:**135–148.
40. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science **270:**1491–1494.
41. Keyes LA, Moore LG, Walchak SJ, Dempsey EC 1996 Pregnancy-stimulated growth of vascular smooth muscle cells: Importance of protein kinase C-depencent synergy between estrogen and platelet-derived growth factor. J Cell Physiol **166:** 22–32.
42. Mosselman S, Poman J, Dijkema R 1996 ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett **392:**49–53.
43. Paech K, Webb P, Kuiper GJM, Nilsson S, Gustafsson J-A, Kushner PJ, Scanlan TS 1997 Differential ligand activation of estrogen receptors ER-α and ER-β at AP1 sites. Science **277:** 1508–1510.

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Received in original form July 7, 1997; in revised form September 25, 1997; accepted October 31, 1997.