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Further Characterization of Human Fetal Osteoblastic hFOB 1.19 and hFOB/ERα Cells: Bone Formation In Vivo and Karyotype Analysis Using Multicolor Fluorescent In Situ Hybridization

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Abstract   We have previously generated an immortalized human fetal osteoblastic cell line (hFOB) using stably transfected temperature sensitive SV40 T-antigen (Harris et al. [1995a] J. Bone. Miner. Res. 10:178–1860). To characterize these cells for phenotypic/genotypic attributes desired for a good cell model system, we performed karyotype analysis by multicolor fluorescent in situ hybridization (M-FISH), their ability to form bone in vivo without developing cell transformation, and finally their ability to form extracellular matrix formation in vitro. The karyotype analysis of hFOB cells revealed structural or numeric anomalies involving 1–2 chromosomes. In contrast, the human osteosarcoma MG63 cells displayed multiple, and often complex, numeric, and structural abnormalities. Subcutaneous injection of hFOB cells in the presence of Matrigel into nude mice resulted in bone formation after 2–3 weeks. Electron microscopic analysis of the extracellular matrix deposited by hFOB cells in culture revealed a parallel array of lightly banded fibrils typical of the fibrillar collagens such as type I and III. These results demonstrate that the hFOB cell line has minimal chromosome abnormalities, exhibit the matrix synthetic properties of differentiated osteoblasts, and are immortalized but non-transformed cell line. These hFOB cells thus appear to be an excellent model system for the study of osteoblast biology in vitro. J. Cell. Biochem. 87: 9–15, 2002.   © 2002 Wiley-Liss, Inc.

Key words: osteoblasts; differentiation; hFOB cells; hFOB/ER cells; MG63 cells; karyotype analyses; multiprobe FISH; in vivo bone formation; matrix production

The availability of good model cell lines for studies of human osteoblast function, differentiation, regulation by hormones, and mechanical stress, etc., have been limited. Of the osteoblast model systems that have been established to characterize osteoblast growth and differentiation in vitro, the two that are widely used are primary cultures obtained from human bone fragments and osteosarcoma derived cell lines generated from human bone tumors. The primary osteoblast cultures are an excellent normal model system but they have their limitations due to heterogeneity of phenotype and stage of differentiation, a slow growth rate, and limited life span in culture. Several transformed human osteosarcoma cell lines are used as alternatives to primary cultures, including MG63 line [Heremans et al., 1978], SaOS-2 [Fogh et al., 1977], U2-OS [Poten and Saksela, 1967], and TE-85 cells [McAllister et al., 1971]. Although these cell lines are more homogenous, they usually do not exhibit the complete phenotype of differentiated osteoblasts, have abnormal growth properties including loss of cell contact inhibition, and exhibit responses to hormones and cytokines that sometimes differ

from those of primary cultures. Even though the osteosarcoma cells can be grown in culture indefinitely, their tumor origin and properties render them questionable as representative of normal osteoblasts [Clover and Gowen, 1994].

To overcome this problem, we established a human fetal osteoblastic cell (hFOB 1.19) line by stably transfecting fetal bone-derived osteoblast cells with a temperature-sensitive mutant (tsA58) of the SV40 T antigen [Harris et al., 1995a]. These cells are known to express many osteoblastic markers including high alkaline phosphatase activity, 1,25 dihydroxyvitamin D_3 inducible osteocalcin expression, and parathyroid hormone (PTH) inducible cAMP production [Harris et al., 1995a]. The hFOB cells have also been shown to spontaneously produce mineralized nodules in in vitro culture. Since these cells express undetectable levels of estrogen receptors (ER), we stably transfected the parental hFOB cells with human estrogen receptor-α gene to generate several clonal cell lines, which express different levels of receptors of ERα [Harris et al., 1995b]. The parental hFOB and the hFOB/ERα cell lines are currently being used in an increasing number of laboratories around the world to study the regulation of osteoblast growth and differentiation and the responses of human osteoblasts to the anabolic hormone, estrogen.

To further characterize the parent hFOB cells, we have analyzed in this article, (1) the karyotype of the hFOB cells compared to MG63 osteosarcoma cells using a new multicolor fluorescent in situ hybridization (M-FISH) procedure; (2) the ability of the cells to form bone tissue in vivo following injection of hFOB cells into nude mice without developing tumors; and (3) the ultrastructure of the extracellular matrix deposited by hFOB cells in vitro using electron microscopy.

MATERIALS AND METHODS

hFOB Cell Cultures

The generation of the immortalized hFOB cell line has been described previously [Harris et al., 1995a]. The mutant SV40 TAg gene (tsA58) used in the immortalization, generates a functional SV40 protein that adopts a functional conformation at 34°C, while at 39°C the TAg becomes unstable and non-functional. Thus, the cells divide rapidly at 34°C (when the TAg is functional) but cease dividing at 39°C (when the TAg is inactive) and start to differentiate and produce mineralized nodules.

The hFOB cells were routinely maintained in Dulbecco's minimal essential medium/Ham's F12 [DMEM/F12-1:1 (w/w) mix] containing 10% (v/v) fetal bovine serum (FBS) and 300 µg/ml neomycin (G418). The hFOB cells stably transfected with a human estrogen receptor-α gene (hFOB/ER9) were maintained in DMEM/F12 containing 10% (v/v) FBS, neomycin (300 µg/ml) and hygromycin (150 µg/ml). The MG63 human osteosarcoma cell line was maintained in DMEM/F12 containing 10% (v/v) FBS.

Assay for In Vivo Bone Formation in Nude Mice

The hFOB cells for injection were cultured in DMEM/F12 at 34°C until confluent, harvested in trypsin/EDTA and washed three times in phosphate buffered saline (PBS). Cells were resuspended in PBS or Matrigel at a concentration of 10^7 cells/ml. The dorsal surface of the nude mice was prepared with Betadine and 70% (v/v) alcohol. Twelve mice each were injected subcutaneously with either of the following: (1) 0.5 ml PBS (as a negative control), (2) 5×10^6 FOB cells in 0.5 ml PBS, (3) 5×10^6 FOB cells in 0.5 ml Matrigel, (4) 5×10^6 FOB cells in 0.5 ml PBS containing 150 mg Collagraft [a commercially available mixture of fibrillar (type I bovine) collagen together with porous beads composed of 60% hydroxyapatite and 40% (w/v) tricalcium phosphate ceramic that is used clinically as a bone graft substitute]. This mixture was implanted through a small stab wound in the dorsal skin, which was then closed with a single skin staple. Four animals from each group were sacrificed at 7, 14, and 21 days after implantation. Bone formation in the implants was evaluated histologically. Multiple 6 µm thick sections from each implant were stained with hematoxylin and eosin, or with Mason's trichrome stain. Bone tissue was identified by light microscopy for the presence of mineralized and unmineralized matrix and by the presence of osteocytes located in lacunae.

Multicolor Fluorescence In Situ Hybridization (M-FISH) Analyses for Detection of Chromosomal Abnormalities

The M-FISH analysis was performed on cultured hFOB, hFOB/ER9, and MG63 cells using

standard protocols for harvesting and slide presentation. The slides were artificially aged for 1–2 min in a dry HYBrite (Vysis, Downers Grove, IL) at 90°C, flooded with $2 \times$ SSC for 1–2 min and dehydrated in 70 (v/v), 85 (v/v), and 100% (v/v) ethanol at room temperature for 1 min in each concentration. The slides were destained in methanol and glacial acetic acid fixative (3:1 v/v) for 10–15 s and jet air dried. The slides were then placed sequentially in $2 \times$ SSC for 5–15 min at 37°C, 1% (v/v) formaldehyde in PBS for 5 min in room temperature, balanced phosphate buffer (PBS) for 5 min at room temperature, and dehydrated in 70 (v/v), 85 (v/v), and 100% (v/v) ethanol for 1 min each at room temperature. Ten microliters of an M-FISH probe was placed on the hybridization site, coverslipped, and sealed with rubber cement and placed in HYBrite with a setting of: melting temperature 80°C, melting time 3 min, hybridization temperature 37°C, hybridization time 16–20 h.

After hybridization, the slides were washed in $0.4 \times$ SSC at 70°C for 2 min, rinsed in tap water and $2 \times$ SSC/0.1% (v/v) Nonidet P-40 at room temperature for 5–30 s, jet air dried, and counterstained with DAPI (100 ng DAPI/ml of antifade). Slides were analyzed with a $40 \times$ objective (Zeiss microscope) using the spectrum gold filter. The image was captured at $100 \times$ magnification using software for M-FISH (Vysis), wherein the image of all five fluorophores and DAPI counterstain were individually captured and merged into one image in which each chromosome is classified to a predefined fluorophore signature. At least five metaphases were analyzed by M-FISH and the results were consistent for each of the three cell lines described.

Transmission Electron Microscopy of the hFOB Cell Matrix Formation and Mineralization

The hFOB cells were seeded on to fibronectin-coated Aclar plastic and grown to confluency at 34°C in medium supplemented with ascorbic acid (50 μg/ml) and β-glycerophosphate (4 mM). At confluency cells were transferred to 39°C and cultured for a further 14 days so that the cultures became multi-layered and the cells fully differentiated. The cells were fixed in 4% (w/v) paraformaldehyde, 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, rinsed extensively in phosphate buffer and post-fixed in 1% osmium tetroxide. Specimens were dehydrated in graded ethanols and 100% propylene oxide and embedded in Spurr's resin. Ultra-thin sections cut perpendicular to the culture surface and placed on 200 mesh copper grids were stained with 2.5% uranyl acetate in absolute ethanol and 0.2% aqueous lead citrate. Electron micrographs were taken on a JEOL 1200 EXII microscope at 60 kV.

RESULTS

The hFOB cells have previously been reported to produce and mineralize a bone-like matrix. Although the hFOB cells exhibit several OB characteristics in vitro, including deposition of a mineralized extracellular matrix [Harris et al., 1995a], the most stringent criteria for an osteogenic cell is whether it is capable of forming bone tissue in vivo. Therefore, the osteogenic nature of hFOB cells was further analyzed by determining whether this immortalized cell line would produce bone following subcutaneous injection into nude mice.

Analysis of In Vivo Bone Formation by the hFOB Cells

When hFOB cells were implanted as a suspension in PBS, the injected material failed to form a mass at the injection site. Other than the skin clip used to mark the injection site, no evidence of the injection was present 4 days after injection and bone formation was not observed on any preparation at any time point (data not shown). When the hFOB cells were implanted with Matrigel, the injected material initially formed a small, flattened mass at the injection site, which became less apparent with time. In contrast to the PBS injections at 14 and 21 days after implantation, bone formation was seen in Matrigel hFOB cell injections in >50% of the implants (See Fig. 1, Panels A, B, and Table I).

Finally, when the hFOB cells were implanted with Collagraft and PBS, a mass readily developed soon after implantation which remained approximately the same size 7 and 14 days after implantation (data not shown). Evidence for resorption of the Collagraft was seen beginning 7 days after implantation, and there was a general decrease in the size of the implant from 14 to 21 days. There was no evidence of bone formation in the Collagraft/PBS/hFOB cell injections (data not shown).

A
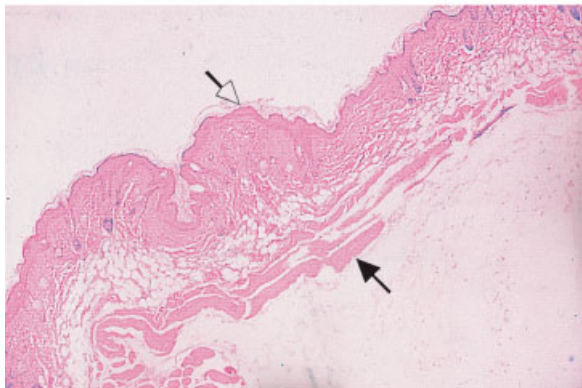
B
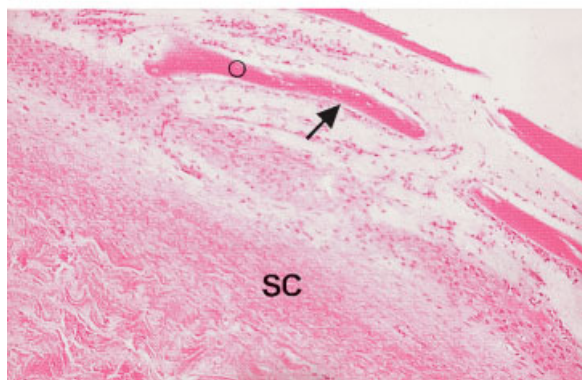
Fig. 1. Photomicroscopy of hFOB cell subcutaneous injection sites in nude mice. (**Panel A**) Subcutaneous tissue and overlying skin 21 days after implantation from an animal injected with 5×10^6 FOB cells in PBS. No evidence for bone formation is seen in the space between the skin and muscle (25 diameters original magnification). Closed arrow shows the subcutaneous muscle and the open arrow shows the skin surface. (**Panel B**) Subcutaneous tissue 21 days after implantation from an animal injected with 5×10^6 FOB cells in Matrigel. Flattened spicules of bone are seen below a thickened subcutaneous tissue (250 diameters original magnification). The open circle shows the osteocyte lacunae, the closed arrow shows the bone spicule. SC, subcutaneous tissue.

TABLE I. Development of Bone In Vivo Using hFOB Cells Implanted With Matrigel

Day after implantation	Number of animals	Number of animals showing subcutaneous bone formation
7	4	0
14	4	3
21	4	4

## Electron Microscopy of Extracellular Matrix Deposited by hFOB Cells In Vitro

As shown in Figure 2, day 14 post-confluent cultures of hFOB cells contained multiple cell layers separated by an extracellular martix composed of bundled fibrils, irregularly arranged thinner fibrils, and vesicles some of which contained electron-dense material. The fibrils in parallel array were lightly banded typical of fibrillar collagens including collagen type I and III. There was little evidence of mineralization within the extracellular space. The cells possessed oval shaped nuclei and contained an extensive network of endoplasmic reticulum, Golgi apparatus, and numerous membrane-bound vesicles and vacuoles of varying density. The predominance of these organelles is a characteristic of cells involved in active secretion. Also within the cytoplasm were abundant mitochondria, and microfilaments were observed adjacent to the cell surface.

M-FISH Analyses of the hFOB Cell Line

It was of interest to compare the karyotype between hFOB cells and MG63 cells as a representative osteosarcoma-derived cell line, using a new and sensitive, multicolor FISH technique.

Speicher et al. [1996] used five fluorophores to assign a unique fluor combination to each of the 22 autosomes, X and Y chromosomes. By use of specific filterset and computer software it was possible to visualize each of the 24 chromosomes in a unique color by this process of multicolor
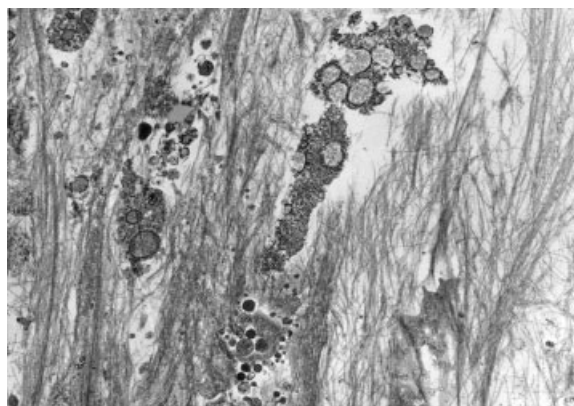

Fig. 2. Electronmicrograph of the nodules produced by hFOB cells in post-confluent culture. The hFOB cells were cultured at 14 days post-confluency and the cell layers together with surrounding matrix were fixed and photographed as described in Materials and Methods.

fluorescence in situ hybridization (M-FISH). Recently, commercially available M-FISH probes were successfully evaluated in a variety of tissue types [Jalal and Law, 1999]. The technique to optimize M-FISH in clinical practice has been described by Law and Jalal [2000].

M-FISH karyotypic analyses were performed on hFOB, hFOB/ER9 cell lines, and MG63 osteosarcoma cell line because banded chromosome analysis do not identify complex abnormalities. The hFOB cell line had a karyotype of 47,XX, + del(16)(q?) (Fig. 3A). The extra chromosome was identified as a deleted 16 with a breakpoint most likely in the long arm. Interestingly, the M-FISH analyses of hFOB/ER9 cell line, which was derived by parental hFOB cells by stable transfection with estrogen receptor-α gene [Harris et al., 1995b] revealed a karyotype of 46,XX,del(6)(q12) (Fig. 3B). It is also a diploid female cell line with a normal pair of chromosome 16 but a deleted chromosome 6 with a breakpoint at 16q12. An M-FISH karyotype was also performed on MG63 cell line. The karyotype (Fig. 3C) was: 66XXY,+ der(Y)(Y;12), + 1, + 2, + der(3)t(3;21),der(4)t(3; 4); + dup(5), + der(5)t(5;17), + der(6)t(6;17;20),+ der(7)t(7;20) + der(8)t(8;9;18),x2,−9,−9, + 10, + der(11)t(11;16),der(12)t(1;12), + der(12)t(12; 131),der(13)t(5;13), + 15, + der(15)t(15;17),+16, der(17)t(8;17),der(17)t(9;17),+18, + 19,+19,−21, + 22, + 2mar. The extra chromosomes, including 14 derivative chromosomes (two of which were derived from three chromosomes and one was present in duplicate), and a duplication were identified. However, there were two complex markers derived from multiple chromosomes. The one on the left is composed primarily

of chromosome 4 and the one on the right is derived from chromosomes 4, 8, 16, 17, and 21.

By comparing M-FISH karyotype analyses of hFOB, hFOB/ER9, and MG63 cells, it is evident
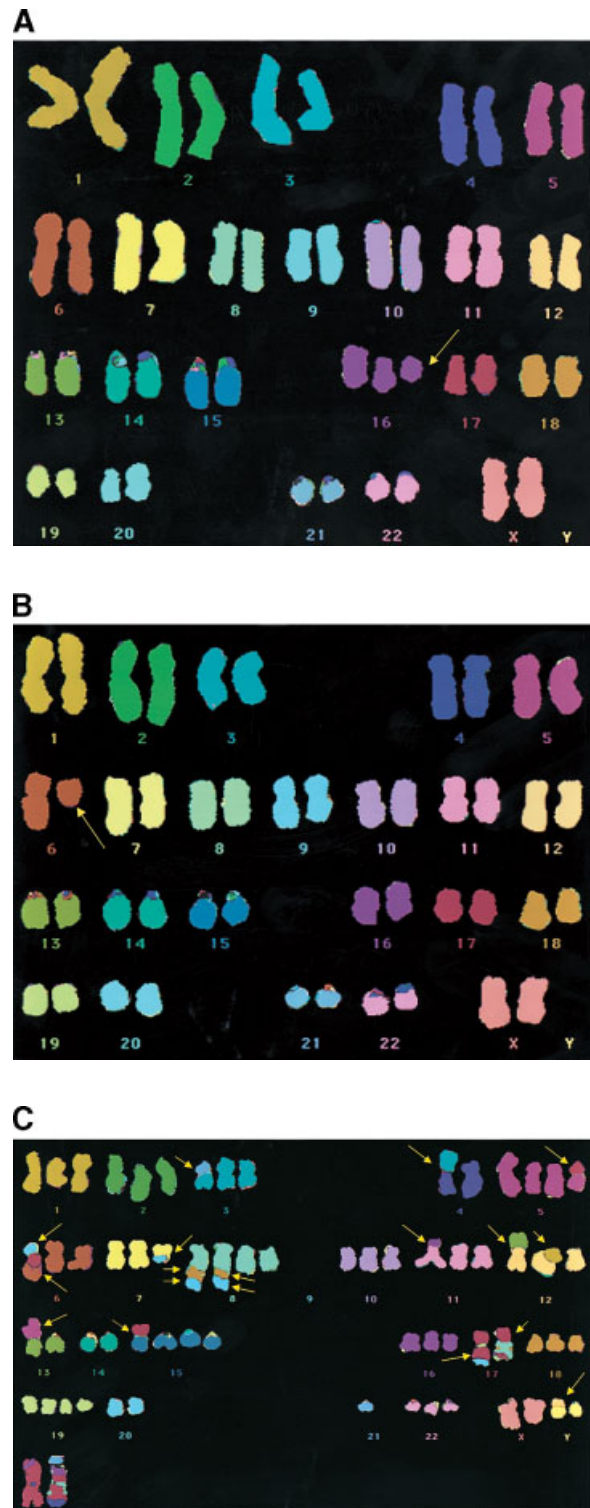
Fig. 3. M-FISH karyotypic analysis was performed on hFOB, hFOB/ER9 cell lines, and MG63 osteosarcoma cell line to identify structural and numeric chromosome anomalies. Many of these abnormalities could not be resolved by standard banded chromosome analysis. The hFOB cell line karyotype was 47,XX, + del(16)(q?). (**A**) Arrow identifies the extra deleted chromosome 16. The hFOB/ER9 cell line had a karyotype of 46,XX,del(6)(q12). (**B**) Arrow marks the deletion chromosome 6. (**C**) The karyotype of MG63 was: 66,XXY, +der(Y)(Y;12), +1, + 2, +der(3) t(3;21),der(4)t(3;4); +dup(5), +der(5)t(5;17), + der(6)t(6;17;20), +der(7)t(7;20) +der(8)t(8;9;18), x 2,−9,−9, +10, +der(11)t(11;16), der(12)t(1;12), +der(12)t(12;13), der(13)t (5;13), +15, +der(15)t(15;17), +16, der(17)t(8;17), der(17)t (9;17), + 18, + 19, + 19, −21, + 22,+2mar. Single arrow marks chromosomes with one exchange, two arrows with two exchanges involving two different chromosomes, and two marker chromosomes with multiple exchanges are presented on the lower left corner.

that hFOB and hFOB/ER9 showed a minimal chromosomal alteration. Minor chromosomal changes such as these are quite common in cell lines that have been cultured in vitro for a long period of time. In contrast, MG63 cells exhibited numerous numeric and complex structural chromosomal abnormalities, that include many derivative chromosomes originating from multiple chromosomes. Consequently, it is evident that hFOB and hFOB/ER9 cells contain far fewer karyotypic alterations and indeed the minor damage detected is, in fact, typical of primary cultured cell lines.

DISCUSSION

The characterization of this cell line described here demonstrates that the hFOB and hFOB/ER9 cell lines are normal (non-transformed) human osteoblasts, with minimal karyotype damage and bone forming capacities. In contrast, the MG63 cells used in many studies are transformed osteoblasts with significant chromosomal abnormalities.

We correlate the formation of bone after implantation with Matrigel to the stability of the implanted mass and the absence of an inflammatory/resorptive response at the implant site. Additionally, attachment factors or growth factors in Matrigel may enhance the expression of the osteoblast phenotype by hFOB cells, as well as reduce migration/dispersal of cells from the implant site. The absence of bone formation after implantation of hFOB cells with PBS is speculated to be due to pressure and movement of the overriding skin resulted in the immediate dispersal of the implanted cells. The Collagraft material was associated with an inflammatory process and this may explain the observed resorption of the mineralized material of this substrate, and may be related to the failure to form bone after implantation of this material.

Previous studies have shown that the hFOB parent cell line displays a 1,25 vitamin D_3 induction in osteocalcin secretion, a PTH induction of cAMP levels, and ceased proliferation and underwent a programmed differentiation to form mineralized nodules after reaching confluence [Harris et al., 1995a]. These properties are similar to those well documented with normal osteoblasts in vivo and primary cultures of osteoblast cells in vitro by the Stein and Lian laboratories [Aronow et al., 1990; Dworetzky

et al., 1990; Stein and Lian, 1993], and other laboratories [Robey and Termine, 1985; Gerstenfeld et al., 1987; Yoon et al., 1987].

In further studies from our laboratories with the hFOB/ER9 cell lines containing the estrogen receptor-alpha isoform, we have shown similar phenotypic changes as reported for normal osteoblast in culture models or in vivo. These changes include E_2 inhibition of IL-6 production [Kassem et al., 1996], E_2 stimulation of both BMP-6 production [Rickard et al., 1998], and OPG mRNA and protein levels [Hofbauer et al., 1999], the E_2 regulation of bone matrix gene expression and mineralization [Harris et al., 1995b] and the E_2 inhibition of osteoblast cell proliferation and differentiation including the regulation of many osteoblast specific genes [Robinson et al., 1997]. Further, the estrogen metabolites and SERMs were also shown to generate a similar response in gene expression [Robinson et al., 2000]. In other laboratories, the hFOB cells have been used as normal osteoblast model system to study the expression of caveolae and caveolin [Solomon et al., 2000a], and characterization of caveolin enriched signaling complexes ("rafts") in the membranes of osteoblasts [Solomon et al., 2000b].

Clover and Gowen [1994] performed a detailed comparison of various osteoblastic markers between MG63 and HOS TE85 cell lines. Whereas, the MG63 cells were found to possess certain properties of normal osteoblastic cells including cell adhesion, osteocalcin expression, and integrin subunit expression, they also displayed abnormal responses with respect to cell proliferation and alkaline phosphatase expression and lack of mineralization of matrix in vitro. The hFOB and hFOB/ER cells display not only normal osteoblast gene expressions, responses to hormones and bone formation, but also controlled cell proliferation (e.g., cell–cell contact inhibition and no transformed phenotype in vivo or in vitro), even after multiple passages in culture.

Our M-FISH analysis suggests that osteoblastic osteosarcoma cell lines, such as MG63, may also exhibit numerous karyotypic alterations (complex structural, and numeric), which may underly some of their atypical characteristics. The immortalized, but non-transformed, hFOB and hFOB/ER9 cell lines, possess very few chromosomal abnormalities. Our demonstration that the hFOB cells can generate a bone-like tissue in vivo secrete an ECM in vitro,

which has ultrastructural elements similar to the ECM deposited by primary osteoblasts in vitro, and do not transform over multiple passages in vitro or in vivo in mice, should make them excellent models in the laboratory.

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REFERENCES

Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB. 1990. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol 143:213–221.

Clover J, Gowen M. 1994. Are MG63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? Bone 15:585–591.

Dworetzky SI, Fey EG, Penman S, Lian JB, Stein JL, Stein GS. 1990. Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation. Proc Natl Acad Sci USA 87:4605–4609.

Fogh J, Fogh JM, Orfeo T. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J Natl Cancer Inst 59:221–226.

Gerstenfeld LC, Chipman SD, Glowacki J, Lian JB. 1987. Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev Biol 122:49–60.

Harris SA, Enger RJ, Riggs BL, Spelsberg TC. 1995a. Development and characterization of a conditionally immortalized fetal osteoblastic cell line. J Bone Miner Res 10:178–186.

Harris SA, Tau KR, Enger RJ, Toft DO, Riggs BL, Spelsberg TC. 1995b. Estrogen response in the hFOB 1.19 human fetal osteoblastic cell line stably transfected with the human estrogen receptor gene. J Cell Biochem 59:193–201.

Heremans H, Billiau A, Cassiman JJ, Mulier JC, DeSomer P. 1978. In vitro cultivation of human tumor tissues. II. Morphological and virological characterization of three cell lines. Oncology 35:246–252.

Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL. 1999. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. Endocrinology 140:4367–4370.

Jalal SM, Law ME. 1999. Utility of multicolor fluorescent in situ hybridization in clinical cytogenetics. Genet Med 1:181–186.

Kassem M, Harris SA, Spelsberg TC, Riggs BL. 1996. Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. J Bone Miner Res 11:193–199.

Law M, Jalal SM. 2000. M-FISH technique: How to set up and analyze. J Assoc Genet Tech 26:51–53.

McAllister RM, Gardner MB, Greene AE, Bradt C, Nichols WW, Landing BH. 1971. Cultivation in vitro of cells from human osteosarcoma. Cancer 27:397–402.

Poten J, Saksela E. 1967. Two established in vitro cell lines from human mesenchymal tumors. Int J Cancer 2:434–447.

Rickard DJ, Hofbauer LC, Bonde SK, Gori F, Spelsberg TC, Riggs BL. 1998. Bone morphogenetic protein-6 production in human osteoblastic cell lines: Selective regulation by estrogen. J Clin Invest 101:413–422.

Robey PG, Termine JD. 1985. Human bone cells in vitro. Calcif Tissue Int 37:453–460.

Robinson JA, Harris SA, Riggs BL, Spelsberg TC. 1997. Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919–2927.

Robinson JA, Waters KM, Turner RT, Spelsberg TC. 2000. Direct action of naturally occurring estrogen metabolites on human osteoblastic cells. J Bone Miner Res 15:499–506.

Solomon KR, Adolphson LD, Wank DA, McHugh KP, Hauschka PV. 2000a. Caveolae in human and murine osteoblasts. J Bone Miner Res 15:2391–2401.

Solomon KR, Danciu TE, Adolphson LD, Hecht LE, Hauschka PV. 2000b. Caveolin-enriched membrane siganling complexes in human and murine osteoblasts. J Bone Miner Res 15:2380–2390.

Speicher MR, Ballard SG, Ward DC. 1996. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet 12:368–375.

Stein GS, Lian JB. 1993. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14:424–442.

Yoon K, Buenaga R, Rodan GA. 1987. Tissue specificity and developmental expression of rat osteopontin. Biochem Biophys Res Commun 148:1129–1136.