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Serial Passage of MC3T3-E1 Cells Alters Osteoblastic Function and Responsiveness to Transforming Growth Factor-β1 and Bone Morphogenetic Protein-2

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The murine-derived clonal MC3T3-E1 cell is a well-studied osteoblast-like cell line. To understand the effects of serial passages on its cellular function, we examined changes in cell morphology, gap junctional intercellular communication (GJIC), proliferation, and osteoblastic function between early passage (<20) and late passage (>65) cells. MC3T3-E1 cells developed an elongated, spindle shape after multiple passages. Intercellular communication decreased significantly (33%) in late vs. early passage cells. Transforming growth factor-β1 (TGF-β1) stimulated cell proliferation in early passage cells and induced c-*fos* expression, while it inhibited proliferation in late passage cells. Using alkaline phosphatase (ALP) activity and osteocalcin (OC) secretion as markers for osteoblastic function and differentiation, we demonstrated that both markers were significantly reduced after multiple cell passages. Bone morphogenetic protein-2 (BMP-2) significantly enhanced ALP activity and OC secretion in early passage cells while TGF-β1 exerted an opposite effect. Both BMP-2 and TGF-β1 had minimal effects on late passage cells. We conclude that serial passage alters MC3T3-E1 cell morphology, and significantly diminishes GJIC, osteoblastic function, TGF-β1-mediated cell proliferation, and responsiveness to TGF-β1 and BMP-2. Cell passage numbers should be clearly defined in functional studies involving MC3T3-E1 cells.

The clonal MC3T3-E1 cell was derived from newborn mouse calvaria and is a well-studied osteoblast-like cell line (1). These cells have been shown to exhibit a developmental sequence typical for osteoblasts and have been suggested as an *in vitro* model of osteoblast differentiation and maturation (2). Quarles *et al.* showed that MC3T3-E1 cells plated at 5,000 cells/cm^2 undergo marked cell replication without expression of alkaline phosphatase (ALP) or accumulation of mineralized extracellular collagenous matrix during days 1–9 in culture (2). During days 9–16, they undergo osteoblastic differentiation that is characterized by high ALP activity (3), osteocalcin (OC) secretion (4), and responsiveness to parathyroid hormone (5). After day 16, the mineralization of extracellular matrix marks the final phase of osteoblast phenotypic development (2). Several experimental variables affect the differentiation of MC3T3-E1 cells and their responsiveness to growth factors, including the original stage of differentiation, cell passage number, source of serum, and plating density (6, 7).

Transforming growth factor-βs (TGF-βs) and bone morphogenetic proteins (BMPs) are regulators of bone repair and regeneration and promote osteogenesis *in vivo* (8, 9). The mechanisms underlying their stimulatory effects have not yet been fully defined. BMP is osteoinductive both *in vivo* and *in vitro* (9, 10, 11) and stimulates osteoblast functional markers, including ALP activity, OC secretion, and type I collagen synthesis *in vitro* (12). Reports on the effects of TGF-β1 on osteogenesis have been conflicting (13, 14, 15). *In vitro* studies show that the effects of TGF-β1 on bone formation, osteoblast differentiation and function differ according to the cell type, species, and other experimental conditions. However, most studies report that TGF-β1 inhibits bone morphogenesis (12, 16, 17).

In our previous study, we reported that TGF-β1 and BMP-2 inhibited gap junctional intercellular communication (GJIC) in MC3T3-E1 cells (18). At that time we observed a decrease in GJIC in untreated control groups with higher passage numbers compared to

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lower passage numbers. In this study, we attempted to define passage-dependent changes in MC3T3-E1 cell function. We report passage-dependent changes in MC3T3-E1 cell morphology, intercellular communication, cell proliferation, osteoblastic function, and responsiveness to TGF-β1 and BMP-2.

MATERIALS AND METHODS

Materials. Mouse MC3T3-E1 cell line and recombinant human BMP-2 were generous gifts from Dr. G. Rodan (Merck, Sharpe and Dohme, West Point, PA) and Dr. Gerard Reidel (Genetic Institute, Cambridge, MA), respectively. Purified human TGF-β1 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). ^{3}H-thymidine (6.7 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Mouse ^{125}I-osteocalcin kit was purchased from Bio-Technology Inc. (Waltham, MA). Restriction enzymes, *Taq* polymerase, dNTPs, and oligo dT primers were purchased from Promega (Madison, WI). Oligonucleotides for polymerase chain reactions (PCR) were synthesized by Bioserve Inc. (Laurel, MD). Primer pairs for c-*fos* and β-actin were purchased from Clontech (Palo Alto, CA). Superscript II RNase H-reverse transcriptase was obtained from Gibco Life Technologies (Gaithersburg, MD).

Cell culture. MC3T3-E1 cells were plated at 5,000/cm^2 on plastic 75 cm^2 culture flasks (Costar, Cambridge, MA) in α-MEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 26 mM $NaHCO_3$, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, in humidified 5% CO_2/95% air at 37°C. Cells were passaged every 3–4 days after releasing with 0.002% pronase E before reaching confluency. The cell culture started from frozen stock was designated passage number one. Early passage was defined as $<$20 and late passage as $>$65. Cell developmental phases were defined as proliferative (days 1–3 in culture) and differentiated (days 12–14 in culture). Cells in treatment groups were exposed to TGF-β1 and BMP-2 dissolved in phosphate-buffered saline (PBS) containing 4 mM HCl and 0.1% bovine serum albumin.

Gap junctional intercellular communication studies. Using data from our previous study (18), the effects of serial passage on gap junctional intercellular communication (GJIC) were examined. Cell coupling studies were performed with a microinjection technique as previously reported (18, 19). Data from untreated control groups with known passage number were statistically analyzed.

Changes in gap junctions were also studied by measuring Connexin 43 (Cx43) mRNA expression using a RT-PCR assay. Briefly, early and late passage cells were plated, cultured, and harvested on day 3 (proliferative phase) or day 14 (differentiated phase). Total RNA was extracted for RT-PCR with Cx43 and β-actin (control) primers as described below.

Cell proliferation assay. MC3T3-E1 cell proliferation was measured by ^{3}H-thymidine incorporation as previously described (18, 20). Briefly, cells were plated at 3.2×10^3 cells/well into 96-well plates to ensure that cells would be in a linear log growth phase by days 2 and 3 after seeding. Fresh media containing no growth factors (control), TGF-β1 (0.5–32 ng/ml), or BMP-2 (12.5–800 ng/ml) was added on day 2 for a 48 h incubation at 37°C. During the last 6 h of the incubation period, cells were labeled with 0.4 μCi/well of ^{3}H-thymidine. The cells were washed with cold PBS, followed by 5% TCA, absolute ethanol, and then incubated with 0.5N NaOH. Samples were placed in vials and covered with scintillation fluid. Radioactive ^{3}H-thymidine was measured as counts per minute (cpm) with a liquid scintillation counter. The effects of TGF-β1 (2 ng/ml) and BMP-2 (50 ng/ml) on early passage cell proliferation, in either the proliferative or differentiated phase of development were studied with a similar assay.

To measure TGF-β1-mediated c-*fos* expression, MC3T3-E1 cells were treated with TGF-β1 (2 ng/ml) for 1 h during the proliferative phase (day 3) and the differentiated phase (day 14). The cells were harvested for RNA extraction and then RT-PCR was performed with c-*fos* primers as described below.

Osteoblast functional studies. The effects of cell passage on osteoblastic function were examined by measuring alkaline phosphatase (ALP) activity and osteocalcin (OC) secretion. Early and late passage MC3T3-E1 cells were treated with TGF-β1 (2 ng/ml) or BMP-2 (50 ng/ml) for 48 h during the proliferative phase (days 1–3) and harvested on day 14. Cell and media samples were used for the ALP assay and OC radioimmunoassay (RIA), respectively, described below.

The pattern of ALP activity and OC secretion in early passage cells was defined by harvesting cells on days 3, 7, 10, 14, 21, and 28 after treatment with TGF-β1 (2 ng/ml) or BMP-2 (50 ng/ml) for 48 h during the proliferative phase. Cell and media samples were used for the ALP assay and OC RIA, respectively. In addition, total RNA was extracted from these groups to examine the effects of TGF-β1 and BMP-2 on the steady state expression of ALP and OC mRNA using RT-PCR as described below.

Alkaline phosphatase (ALP) assay. MC3T3-E1 cells were grown in 12-well plates, rinsed twice with ice cold phosphate buffered saline (PBS, pH 7.4), solubilized in Tris/glycine/Triton buffer (pH 10.5, 50 mM Tris, 100 mM glycine, and 0.1% Triton X-100), and sonicated on ice with two 15-s pulses. This mixture was centrifuged at 5,000 $\times$ g for 15 min at 4°C, and the supernatant was collected. 100 μl of freshly prepared p-nitrophenyl-phosphate (PNPP) substrate (8,000 μg/ml) was added to 200 μl of the supernatant mixed with Tris/glycine buffer (pH 10.5, 50 mM Tris, 100 mM glycine) in 12 $\times$ 10 tubes and incubated for 30 min at 37°C. The enzymatic reaction was terminated with the addition of 3 ml of ice cold 0.1 N NaOH solution. The optical density (OD) of p-nitrophenol (PNP) at 410 nm was measured with spectrophotometry within one hour. ALP activity was standardized as nmoles of PNP per mg of protein per minute.

Osteocalcin (OC) radioimmunoassay (RIA). Culture media was collected from flasks or multi-well plates, aliquoted into microfuge tubes, and stored at $-$80°C. 50 μl of appropriately diluted samples were mixed with 150 μl of RIA buffer, 50 μl ^{125}I-osteocalcin (10,000 cpm/tube) and rabbit anti-mouse OC antibody (1:200) and incubated at room temperature overnight (16–20 h). After the addition of 1 ml donkey anti-rabbit IgG (1:50) and 2.5% polyethylene glycol (PEG), the reaction was incubated at 4°C for 2 h, mixed briefly, and centrifuged at 5,000 $\times$ g at 4°C for 30 min. The supernatant was discarded, and the radioactivity in the pellets was measured with a gamma counter.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was prepared using an acid guanidinium isothiocyanate-phenol-chloroform extraction method (21). The RNA was dissolved in nuclease-free water and its concentration was estimated by optical density (OD) at 260 nm. 1–5 μg of total RNA plus 0.5 μg of oligo dT was denatured at 70°C for 10 min, chilled on ice, and then preincubated at 42°C for 2 min after adding 10 mM dithiothreitol (DTT), 2.5 mM each of dNTPs, and reaction buffer. 200 units of Superscript II reverse transcriptase was added and incubated at 42°C for 50 min. The reaction was heated to 70°C for 15 min and any cRNA was removed with the addition of 2 units RNase H at 37°C for 20 min.

10% of the RT product was added to a PCR reaction which included PCR buffer (pH 8.4, 20 mM Tris, 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 mM primers, and 5 units *Taq* DNA polymerase. 30 PCR cycles were followed by denaturation at 95°C and extension at 72°C. Sense and anti-sense primers for ALP, OC, and Cx43 were designed with the MacVector program using sequences from a NCBI Blast search (Table 1). c-*fos* and β-actin primers were purchased. PCR products were confirmed with 1% agarose gel electrophoresis. Band density was standardized with the β-actin product and quantified by densitometry.

TABLE 1

Oligonucleotide Primers for RT-PCR

Gene of interest	Oligonucleotides	Fragment size (bp)
Alkaline phosphatase (ALP)	5′-CCAAGACGTACAACACCAACGC-3′ 5′-AAATGCTGATGAGGTCCAGGC-3′	474
Osteocalcin (OC)	5′-AAATGCTGATGAGGTCCAGGC-3′ 5′-ACCGTAGATGCGTTTGTAGGCG-3′	323
Connexin 43 (Cx43)	5′-GTCAGCTTGGGGTGATGAACAG-3′ 5′-ATGGTTTTCTCCGTGGGACG-3′	498
c-*fos*	5′-GAGCTGACAGATACACTCCAAGCG-3′ 5′-CAGTCTGCTGCATAGAAGGAACCG-3′	432
β-actin	5′-GTGGGCCGCTCTAGGCACCAA-3′ 5′-CTCTTTGATGTCACGCACGATTTC-3′	540

Statistical analysis. Mean ± S.D. was calculated from each of two or more independent experiments. One way analysis of variance (ANOVA) followed by Student's *t* test was used to determine significance ($p < 0.05$).

RESULTS

Effects of Serial Passage on Cell Morphology

MC3T3-E1 cells demonstrated marked differences in morphology between early (<20) and late passage (>65) groups (Fig. 1). Early passage cells that were plated and viewed on day 7 remained round while late passage cells viewed on day 7 developed an elongated, spindle shape.

Gap Junctional Intercellular Communication Studies

GJIC is mediated by the transfer of small molecules (<1.5 kD) through transmembrane channels called gap junctions. Intercellular communication can be demonstrated by the transfer of low molecular weight dyes, such as lucifer yellow, through these channels. Retrospective analysis of lucifer yellow dye coupling data revealed 64.6 ± 13.2% coupling in early passage cells (Fig. 2). Late passage cells demonstrated only 43.5 ± 10.7% coupling, a 33% decrease in GJIC ($p < 0.05$).

Connexin 43 (Cx43) is a major component of gap junctions. RT-PCR revealed no significant difference in Cx43 mRNA expression between early and late passage cells whether in the proliferative (day 3) or differentiated (day 14) phase of development (data not shown).

Cell Proliferation Studies

^{3}H-thymidine incorporation assays were performed to study the effects of serial passage on cell prolifera-

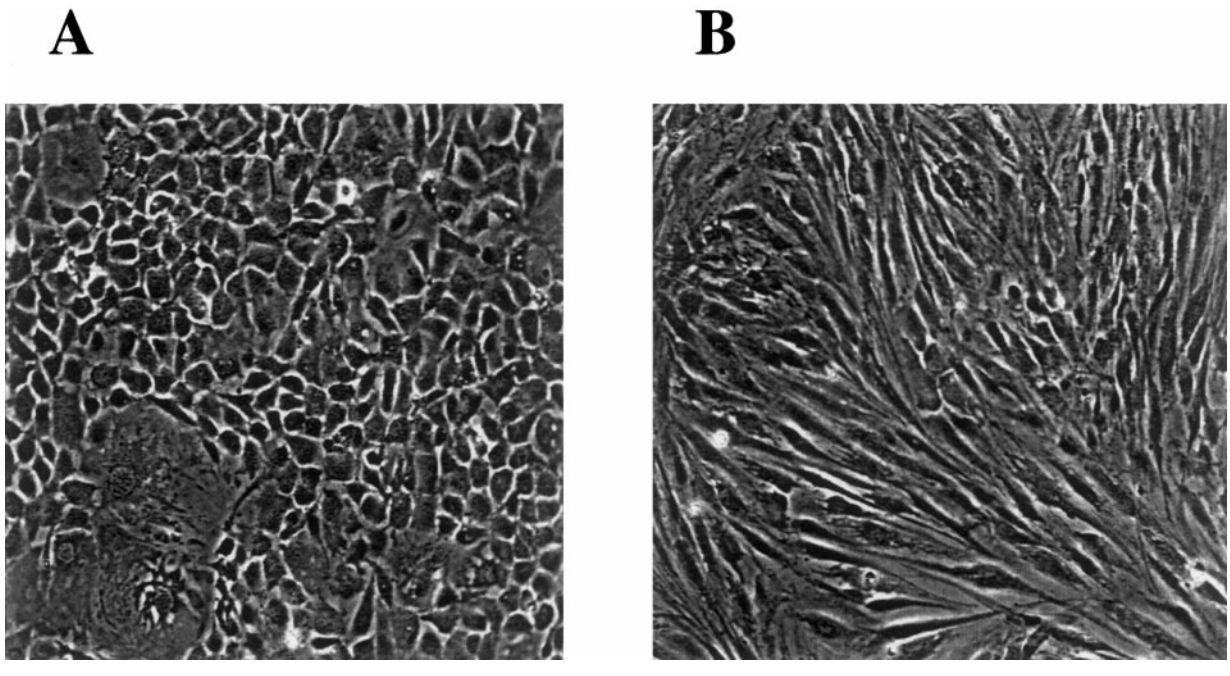

FIG. 1. Effects of serial passage on cell morphology. (A) Early passage (<20) and (B) late passage (>65) MC3T3–aE1 cells photographed on day 7 in culture.

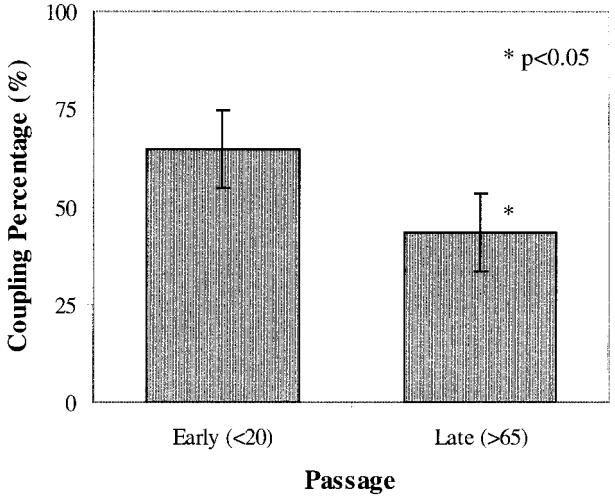

FIG. 2. Effects of serial passage on gap junctional intercellular communication. GJIC (measured as coupling percentage per dye injection) was 64.6 ± 13.2% and 43.5 ± 10.7% in early and late passage cells, respectively. Values are mean ± S.D. of 4 individual injections. (*$p < 0.05$).

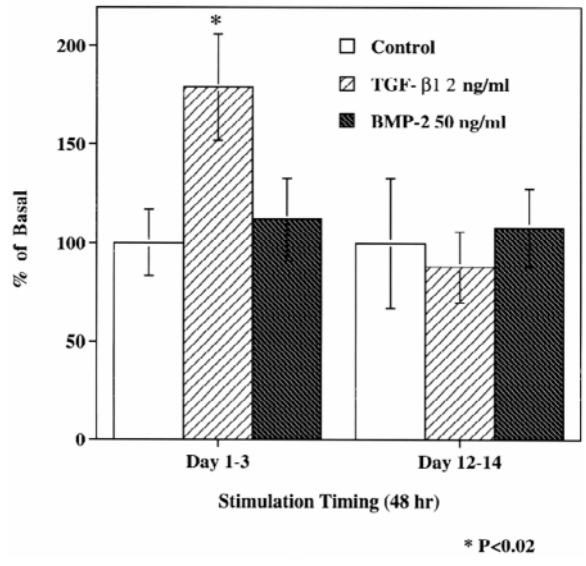

FIG. 3. Effects of cell differentiation on growth factor-mediated cell proliferation. ^{3}H-thymidine incorporation in early passage cells treated with TGF-β1 (2 ng/ml) or BMP-2 (50 ng/ml) during the proliferative (days 1–3) or differentiated phase (days 12–14). Values are mean ± S.D. of 10 replicates with control set at 100%. (* $p < 0.02$, compared to control).

tion. ^{3}H-thymidine uptake was unchanged in untreated MC3T3-E1 cells between early (15320 ± 2051 cpm/well) and late passage groups (15093 ± 4059 cpm/well). ^{3}H-thymidine uptake increased with TGF-β1 treatment in early passage cells and decreased in late passage cells (data not shown). There was no significant effect by BMP-2 on cell proliferation.

TGF-β1 (2 ng/ml) stimulated ^{3}H-thymidine incorporation by 78.5 ± 27.2% ($p < 0.05$, $n = 10$) when added during the proliferative phase, while it had no significant effect when added during the differentiated phase (Fig. 3). BMP-2 (50 ng/ml) had no significant effect on ^{3}H-thymidine uptake during either phase.

In primary cultures of rat osteoblast-like cells, TGF-β1 was shown to induce early gene expression of c-*fos* (16). c-*fos* is a growth factor-inducible immediate-early gene that encodes transcription factors which are thought to activate a program of gene expression essential for cell proliferation (22). In our cells, c-*fos* mRNA expression following TGF-β1 stimulation was clearly detected during the proliferative phase (day 3), but none was detected during the differentiated phase (day 14) (Fig. 4).

Osteoblast Functional Studies

ALP activity and OC secretion were measured to study the effects of serial passage on MC3T3-E1 osteoblastic markers in response to BMP-2 and TGF-β1 stimulation. Timed experiments on early passage cells (data not shown) revealed that basal ALP activity steadily increased up to day 14 (49.1 ± 6.7 mmoles/min/mg protein) and subsequently declined. Basal OC secretion was detectable by day 10, increased up to day 21 (101.1 ± 17.7 μg/mg protein), and remained elevated. Basal ALP activity and OC secretion significantly decreased after multiple cell passages (Fig. 5). BMP-2 significantly enhanced ALP activity and OC secretion in early passage cells but minimally enhanced their levels in late passage cells. TGF-β1 inhibited ALP activity and OC secretion more in early than late passage cells.

ALP mRNA was detectable with RT-PCR by day 3 and subsequently disappeared after day 7. ALP mRNA expression was inhibited by TGF-β1 but not by BMP-2 (data not shown). OC mRNA was highly expressed in a time-dependent manner. OC mRNA expression was inhibited by TGF-β1 and stimulated by BMP-2 (data not shown).

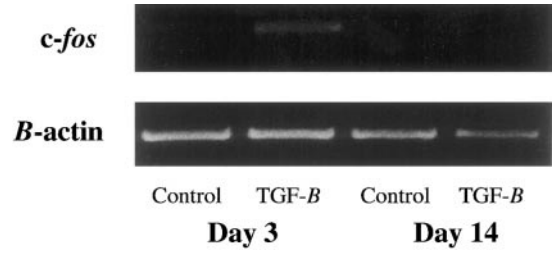

FIG. 4. Effect of TGF-β1 on c-*fos* induction. RT-PCR using c-*fos* primers on total RNA from early passage cells in the proliferative (day 3) or differentiated phase (day 14).

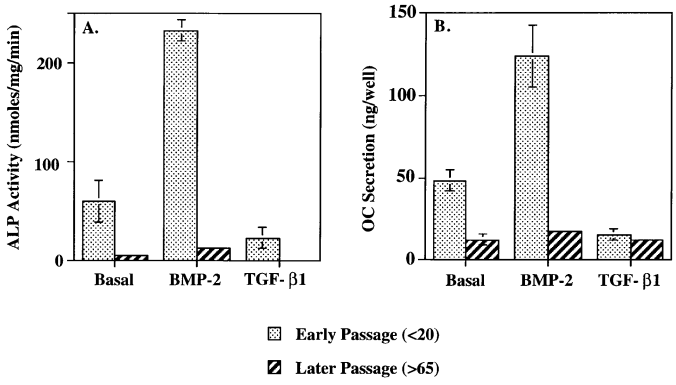

FIG. 5. Effects of serial passage on MC3T3-E1 cell osteoblastic function. (A) ALP assays and (B) OC radioimmunoassays performed on early (dotted bar) and late (striped bar) passage cells treated with BMP-2 (50 ng/ml) or TGF-β1 (2 ng/ml). Values are mean ± S.D. of 4 replicates.

DISCUSSION

The osteoblast-like MC3T3-E1 cell is an established cell line used in many laboratories for osteoblast functional studies. Currently, cell passage numbers are not routinely reported in these experiments. Our study showed significant changes in MC3T3-E1 cell morphology, GJIC, cell proliferation, osteoblastic function, and responsiveness to TGF-β1 and BMP-2 after multiple passages (>65).

MC3T3-E1 cells developed an elongated, spindle shape after serial passages. Interestingly, MC3T3-E1 cells treated with TGF-β1 (2 ng/ml) were reported to develop a similar shape in our previous study (18). An endogenous up-regulation of TGF-β1 or its receptor may be one possible explanation for this morphologic change. Our cells were cultured in the presence of 10% fetal bovine serum, which has been reported to contain a significant amount of TGF-β1 (23). A cumulative effect of TGF-β1 from repeated passage may influence this cell shape. The specific mechanism for this passage-dependent change in cell morphology remains unclear, and is currently under investigation.

Our study showed that MC3T3-E1 cells had significantly reduced intercellular communication after multiple passages. Changes in cell coupling via gap junctions may influence cell proliferation and phenotype. The reduction of GJIC in late passage cells may offer one explanation for their decreased proliferation in response to TGF-β1 and decreased ALP and OC levels. RT-PCR revealed no measurable difference in the steady state level of Cx43 mRNA after serial passage suggesting that a post-transcriptional process may alter the functional cell-to-cell communication in MC3T3-E1 cells.

^{3}H-thymidine incorporation assays after treatment with TGF-β1 showed that cell proliferation increased in early passage cells while it decreased in late passage cells. TGF-β1 has been reported to exhibit a differentiation-dependent biphasic effect on cell proliferation in MC3T3-E1 cells (24). TGF-β1 induced c-*fos* expression in early passage cells during the proliferative phase but not during the differentiated phase. This suggests that TGF-β1 may stimulate MC3T3-E1 cell proliferation by the induction of c-*fos* transcription factors. BMP-2 did not have any significant effects on cell proliferation.

MC3T3-E1 cell osteoblastic function, as measured by ALP activity and OC secretion, decreased after serial passage. BMP-2 significantly enhanced ALP activity and TGF-β1 inhibited its activity in early passage cells. Both growth factors had limited effects on ALP in late passage cells. ALP activity peaked on day 14 and did not appear to be mediated directly by the expression of its mRNA which not detectable after day 7. In other studies using rat primary cultures, ALP mRNA was induced in proliferating cells within an hour of treatment with hydroxyurea (a proliferation inhibitor) but disappeared after 4 h (4). These findings suggest that BMP-2 stimulation of ALP activity occurs at the post-transcriptional level.

BMP-2 significantly enhanced OC secretion in early passage cells but had minimal effect on late passage cells. TGF-β1 inhibited this osteoblastic marker in early passage cells only. OC secretion appeared to be mediated directly by expression of its mRNA, which increased in a time-dependent manner. Other investigators have observed that OC mRNA did not appear until day 6 or day 21 of culture (4, 6). This discrepancy may be explained by the higher sensitivity of RT-PCR in detecting minute quantities of RNA. Northern analysis in our control and BMP-2 treatment groups did not detect OC mRNA expression until day 10.

Aging bone is characterized by a decrease of osteoprogenitor cells (25), uncoupled bone turnover, reduced bone mass and bone formation (26), and decreased responsiveness to hormones and growth factors (27). An age-related decline in metabolic function has also been observed at the cellular level. Several attempts have been made to develop an *in vitro* aging bone model by establishing primary cultures from young and old animals (28). These attempts were limited by the low yield of aged cells but were able to demonstrate diminished cell proliferation and decreased responsiveness to local and systemic growth factors with increasing age (29, 30, 31). Osteoblastic function and cell proliferation were significantly reduced in primary osteoblast-enriched cell cultures from old vs. young subjects (29, 32).

In vitro cellular aging has been studied most extensively with human diploid fibroblast (HDF) cells. Beginning with Hayflick and Moorhead's observation that these cells had a limited doubling capacity, replicative senescence has been interpreted as aging on a cellular level (33, 34). MC3T3-E1 cells do not have a finite

population doubling limit. Senescent HDF cells are larger than early passage HDF cells (35) and their replicative potential has been shown to be inversely related to their early G1 volume (36). Serial passage of MC3T3-E1 cells result in cells of variable size and morphology, which could result from selective passage within a heterogeneous clonal cell population. These findings limit the association between multiple cell passages and *in vitro* cellular aging. It remains unclear whether decreased cellular function and proliferation in serially passaged MC3T3-E1 cells truly reflect aging bone *in vivo.*

It is clear, however, that the cell passage number is an important variable in the proliferative capacity and osteoblastic function of MC3T3-E1 cells. Passage numbers should be clearly defined in functional studies using MC3T3-E1 cells.

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