

EXTRINSIC AND INTRINSIC MECHANISMS OF HUMAN SKELETAL AGING

JULIE GLOWACKI, PHD, AND SHUANHU ZHOU, PHD

DEPARTMENT OF ORTHOPEDIC SURGERY, BRIGHAM AND WOMEN'S HOSPITAL

Bone growth results from the synthetic activity of bone-forming osteoblasts being in excess of osteoclastic resorption (Figure 1). In the adult, skeletal homeostasis is achieved by bone remodeling, a coordinated process in which pockets of bone resorption are balanced by osteoblastic new bone formation (Figure 1). Skeletal aging is characterized as a gradual loss of bone mass due to an excess of bone resorption that is not matched by new bone formation. There are two major types of bone loss, either high (Type I) or low (Type II) turnover, i.e. rates of bone formation and resorption (Figure 1), but both result in net bone loss. Type I (postmenopausal) osteoporosis results from acceleration of bone turnover following hormonal deprivation. In Type II (senile) osteoporosis, there is impairment of osteoblastic bone formation and net low turnover. These can be distinguished by biopsy and by serum biomarkers of formation and resorption, and may benefit from different therapeutic approaches. Regardless of the type, there is a gradual loss of bone mass measured by declining bone mineral density (BMD), to the point of osteopenia or osteoporosis, and bone fracture. Although virtually all individuals show age-related loss of bone mass, it is remarkable that the aged skeleton is capable of robust fracture repair and incorporation of implants, and has capacity for increasing bone mass with exercise, improved nutrition, and other interventions.

Some of the major osteotropic hormones that decrease with age are Growth Hormone (GH), its intermediary, insulin-like growth factor (IGF), and the sex hormones, estradiol (E2), dihydrotestosterone (DHT), and adrenal dehydroepiandrosterone (DHEA). Striking evidence of the effects of estrogen on skeletal mass, for example, is provided by the acceleration of bone loss upon ovariectomy and prevention of that loss by small doses of estrogen. In individuals experiencing the natural menopause or andropause and adrenopause, replacement of sex

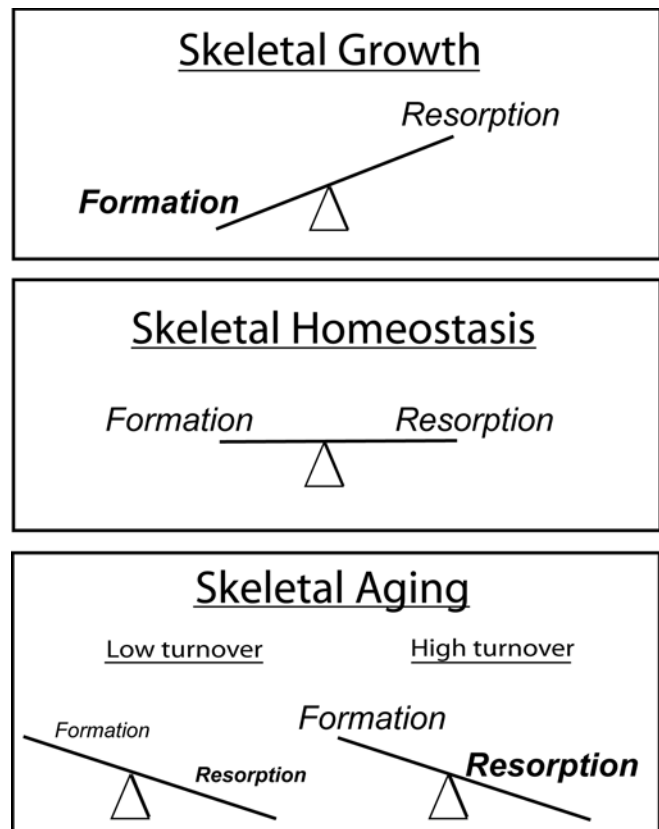


Figure 1. Relative contributions of osteoblastic bone formation and osteoclastic bone resorption in skeletal growth, homeostasis, and aging, including high and low turnover states.

hormones has been shown to mitigate bone loss, but their use holds risk of breast or prostate cancer in susceptible patients. Greater understanding of the mechanisms of skeletal aging is likely to provide new approaches for rejuvenation. Our program of research aims to identify extrinsic and intrinsic mechanisms of skeletal aging in order to discover modifiable pathways for sustaining a robust skeleton. Extrinsic refers to those systemic age-related changes that have an impact on the skeleton, such as endocrinological aging; intrinsic refers to age-related alterations in bone cells that impair their performance (Table 1).

Marrow contains hematopoietic cells and stem cells that give rise to them. In addition, marrow contains a subpopulation of stromal cells that replenish osteoblasts and other connective tissue cells. With extreme old age and osteoporosis, bone marrow becomes fatty and ultimately “gelatinized”. We have used human marrow discarded from men and women

EXTRINSIC	INTRINSIC
Endocrine Estrogen, Testosterone, DHEA, Growth Hormone	Stem/Progenitor Cells Number Rate of Proliferation Response to Mitogens and Differentiation Factors
Nutrition	
Co-Morbidities	Bone Cells Level of Activity Response to Regulation

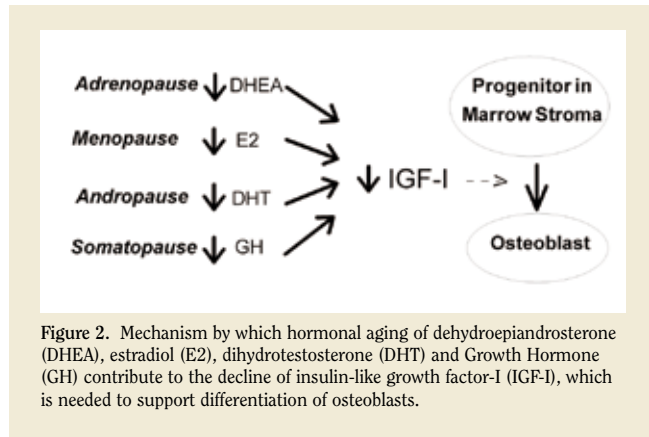


Figure 2. Mechanism by which hormonal aging of dehydroepiandrosterone (DHEA), estradiol (E2), dihydrotestosterone (DHT) and Growth Hormone (GH) contribute to the decline of insulin-like growth factor-I (IGF-I), which is needed to support differentiation of osteoblasts.

undergoing total hip replacement for non-inflammatory joint disease as a precious resource to study human bone cell differentiation. The non-adherent fraction of marrow also includes progenitors of the osteoclast [1], but this review summarizes our recent work on osteoblastogenesis with the adherent fraction of human marrow stromal cells (MSCs). Cultures of MSCs can be used to determine whether age of the subject influences osteoblast differentiation, whether osteoblast differentiation can be stimulated by the hormones that decline with age, and/or whether there is an intrinsic loss or change in osteoblast stem/progenitor cells.

EFFECT OF AGE ON OSTEOBLAST DIFFERENTIATION

There is some discrepancy in the literature about the effect of age on osteoblast differentiation, probably due to imprecisions in methodologies that rely on counting colonies. These assays are difficult to standardize among individual laboratories. Studies that use colony size or number have been criticized because of inappropriate use of parametric statistical methods [2]. Monolayer, two-dimensional colony assays have inherent inaccuracies regarding the volume of cells in each colony and problems with necrosis in the center of the colonies. In addition, some laboratories used vertebral marrow from cadavers and some used iliac crest aspirates. Use of discarded marrow has the additional advantage of providing very large numbers of cells (hundreds of millions) for replicate tests. We used a robust molecular approach and reported that there is an age-related decline in osteoblastogenesis with cultures of human MSCs [3]. This has been shown for marrow obtained from men [3] and women [4].

EFFECT OF OSTEOTROPIC HORMONES TO STIMULATE OSTEOBLAST DIFFERENTIATION

Aging is associated with declines in the sex steroids estrogen, testosterone, and the adrenal androgen DHEA [reviewed in 5]. Estrogens are important for bone growth, maturation, and maintenance in both women and men. Reports of impaired growth and low bone mass in men with mutations in the estrogen receptor or with aromatase deficiency underscore the role of endogenous estrogens in the male skeleton. Some but not all studies show an association between circulating levels of estrogens and bone density in women and in men [5].

Not only do androgen levels decline in men after the age of 40 years, but women also experience an andropause due to declining ovarian synthesis of testosterone. For both men and women, aging of the adrenal gland is restricted to the synthesis of DHEA while serum levels of aldosterone, cortisol, and corticosterone show little change [6].

A unified hypothesis suggests a mechanism by which the menopause, andropause, and adrenopause contribute to age-related bone loss [5]. Changes in these circulating sex hormones may effect dramatic changes in mediators, such as IGF-I, that control the rates of bone formation and bone resorption. Serum levels of IGF-I are inversely correlated with age and with bone density [7]. Synthesis of IGF-I may be modulated by the loss of those hormones and contribute to decreased bone formation (Figure 2).

Given that cultures of marrow stromal cells give rise to osteoblasts when they are grown with osteoblastogenic supplements (10 nM dexamethasone, 5 mM β -glycerophosphate, and 170 μ M ascorbate-phosphate), we tested the effects of osteotropic hormones on marrow from elders in the absence of dexamethasone. For example, MSCs from a 79-year-old woman showed the expected stimulation by dexamethasone (Figure 3), as measured by induction of the osteoblast marker, alkaline

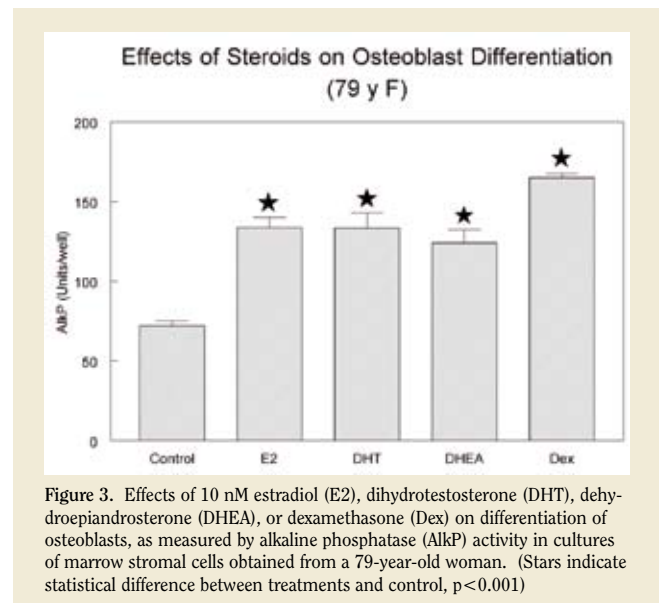


Figure 3. Effects of 10 nM estradiol (E2), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), or dexamethasone (Dex) on differentiation of osteoblasts, as measured by alkaline phosphatase (AlkP) activity in cultures of marrow stromal cells obtained from a 79-year-old woman. (Stars indicate statistical difference between treatments and control, $p < 0.001$)

phosphatase activity, after 6 days of treatment. Physiological concentrations (10 nM) of estradiol (E2), dehydrotestosterone (DHT), or DHEA significantly promoted osteoblast differentiation. Molecular analyses (data not shown) indicated that after 3 days of treatment, each hormone upregulated mRNA for IGF-I, consistent with it being an intermediary in promoting osteoblastogenesis.

INTRINSIC AGE-RELATED CHANGE IN OSTEOBLAST STEM/PROGENITOR CELLS

Whereas age-related changes in circulating levels of hormones are systemic, or extrinsic to the skeleton, we also tested whether there may be intrinsic changes in the osteoblast stem/progenitor cells themselves. This is important for assessing the potential of preventive or therapeutic strategies. Hormone replacement, even with tissue-specific analogs with no carcinogenic risk, will not be adequate if there are fewer stem/progenitors or if their responsiveness to hormones is reduced with age.

Cultures are prepared from discarded marrow by removing differentiated cells by low-speed density centrifugation. Use of a solution with density of 1.077 for human marrow results in the pelleting of heavier differentiated cells, i.e. those with cytoplasmic organelles, such as endothelial cells, red cells, granulocytes, macrophages, mast cells, megakaryocytes, and preosteoblasts and osteoblasts that may have been separated from the bone matrix. Undifferentiated marrow cells with high ratio of nucleus-to-cytoplasm are retained above the solution and can be cultured after washing. This population includes progenitors of hematopoietic lineages, but they are non-adherent and can be readily removed from the adherent stromal population.

We tested the effect of age on proliferation, cell cycling, Senescence-Associated β -galactosidase (SA- β -gal) in cultures from young (<45 years) and older (>55 years) subjects [8]. Cells were counted every other day for calculation of the time

required for population doubling. Samples of MSCs from young and old individuals were examined by flow cytometry for distribution in different stages of the proliferation/resting cycle. SA β -gal-positive cells were enumerated cytochemically. Doubling time of marrow stromal cells was longer in cells from the older (78.8 ± 13.5 hrs) than the younger (48.8 ± 0.7 , $p=0.0011$, Welch t-test) subjects and was correlated with age ($r = 0.736$, $p<0.05$). Cultures of cells from elders could take a longer time to double if they had a prolonged time in resting stage and an equal time for DNA synthesis and mitosis or else they could be prolonged in each of the stages. Flow cytometric analysis showed that there was no significant difference with age in the percent of cells in different stages of cell cycle, but with age, more cells were apoptotic ($r=0.723$, $p = 0.0248$). Finally, there were 4-fold ($p<0.001$) more hMSCs positive for SA β -gal in samples from older than younger subjects. This marker had been shown to reflect *in vivo* and *in vitro* aging of skin fibroblasts [9]. In conclusion, we found an age-related increase in SA- β -gal and apoptosis and a decrease in proliferation of MSCs. These inherent age-related properties of MSCs may contribute to skeletal aging.

DISCUSSION

Human marrow discarded during orthopedic surgery is a precious resource to study human bone cell differentiation. Differentiation to osteoblasts is stimulated by estrogen, testosterone, and DHEA, and, as shown in our other studies, by anabolic doses of PTH and 1,25-dihydroxyvitamin D₃. Our experiments show that there are a number of age-related changes that are manifest *in vitro*, including declines in proliferation and osteoblast differentiation, a prolongation of the stages of the cell cycle, and increases in apoptosis and expression of Senescence-Associated β -galactosidase. Prevention of skeletal aging or rejuvenation of the aged skeleton requires more detailed information about the mechanisms of these differences.

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