

Original Research Article

Impact of estrogen on mechanically stimulated cells in vitro

Jörg Neunzehn^{1*}, Ulrich Meyer² and Hans-Peter Wiesmann¹

¹Max Bergmann Center of Biomaterials and Institute of Materials Science, Technische Universität Dresden, Budapester Str. 27, D-01069 Dresden, Germany

²Maxillofacial Surgery Münster, Schorlemerstr. 26, D-48143 Münster, Germany

*Corresponding author

A B S T R A C T

Keywords

Estrogen,
Osteoblast,
mechanical
stimulation,
bone,
mineralization

Estrogen deficiency and decreased exercise known to be major causes for osteoporosis in elderly patients are assumed on important role in implant failure. Hormone replacement therapy and exercise are established methods to prevent the accompanying bone loss, thereby improving the conditions for implant osseointegration. Whereas the clinical effects of estrogen on bone are well documented, less is known about estrogen effects on loaded and unloaded osteoblasts on a cellular level. This study was aimed at investigating the effects of estrogen on mechanically stimulated osteoblast like cells in culture. Mechanically unstimulated cultures served as controls. Our investigations revealed that estrogen had a suppressive effect on the proliferative response of osteoblasts towards mechanical strain. Estrogen increased the synthesis of bone specific proteins in mechanically stimulated cultures whereas estrogen had no effect on unstimulated cells. The differentiation effects significant altered at estrogen doses of 10nmol and 10µmol. Our data suggest a positive effect of hormone substitution on the composition of the extracellular matrix in loaded bones. In the context of implant dentistry, hormone repainting therapy should be regarded as a medical tool to improve the conditions for an undisturbed implant healing.

Introduction

Implant osseointegration and long term implant success is depended on an undisturbed bone physiology. It is known that load bearing is an important, if not the most important, functional influence on bone mass and architecture. Load-bearing most probably exerts its influence through the dynamic strains engendered in the bone tissue. Mechanically adaptive bone

modeling and remodeling can be therefore regarded as a homeostatic mechanism regulating functional bone strains in the skeleton. Osteoporosis may present a risk factor in achievement of osseointegration (Akca, Sarac et al. 2007). Osteoporosis is a clinical example of failure of this mechanically adaptive mechanism and is characterized by a decline in bone mass,

and presumed consequent increases in functional strain, until the loads incurred during every day activities cause fracture. It is definitively known that androgens and estrogens have an impact on bone formation and bone resorption (Vanderschueren, Gaytant et al. 2008) and that osteoprotegerin is stimulated by estrogen (Gallagher 2008). The findings that bone loss is rapid at the time of estrogen withdraw and adaptive bone gain particularly marked at puberty (Cadogan, Blumsohn et al. 1998; Orbak, Yildirim et al. 1998; Schiessl, Frost et al. 1998), suggests that the mechanically adaptive (re)modeling response is influenced directly or indirectly by the presence of estrogen. Estrogen itself is the most important sex steroid for maintenance of skeletal homeostasis, as reflected in the widespread and beneficial use of estrogen replacement therapy to combat postmenopausal osteoporosis in women. Estrogen and selective estrogen receptor modulators are members of the main classes of anti-resorptives in currently use (Reid 2008). Estrogen functions by binding to and activating nuclear receptors (Bonnelye and Aubin 2005; Heilberg, Hernandez et al. 2005), which then act as transcription factors that induce or repress target gene expression.

It is important to note that estrogen deficiency is not only causing the rapid and transient bone loss that accompanies menopause in women, but also in contributing to the slower, sustained age-related bone loss in elderly women and men (Riggs, Khosla et al. 1998). Early postmenopausal bone loss affects mostly trabecular bone and is the result of increased bone remodeling where the rate of bone resorption out-paces the rate of bone formation, whereas age-related bone loss is caused by indirect consequences of estrogen deficiency.

As implant healing critically depends on an undisturbed bone physiology, estrogen deficiency is occupant to be an important factor in valued in implant failure. Knowledge of underlying cellular mechanics and possible treated regimens are therefore important aspects in implant dentistry. The impact of age related an osteoporosis related bone loss on the maxillofacial skeleton is likely to directly affect the capacity of these bones to integrate endosseous dental implants. Roberts et al for exile the bone responses to dental implants, particularly those factors the optimize implant healing. They found a time-dependent interaction among hormone and biomechanical factors. The suggested bone metabolism appears to have the most profound influence on the resorptive phase of healing; whereas bone formation is mainly controlled by mechanical stimulating (August, Chung et al. 2001; Elsubeihi and Zarb 2001).

Hormone replacement therapy (HRT) is an established pharmaceutical method to prevent postmenopausal osteoporosis (Schlegel, Petersdorf et al. 1999). It also seems that estrogen therapies are useful for avoidance of fractures in perimenopausal women irregardless of the bone conditions (Higuchi, Tarakida et al. 2009). Whereas the clinical effects of HRT are well described, little is known about the close dependency of estrogens on osteoblast in culture, especially in regard to their effects on mechanically induced cell reactions.

To investigate the potential involvement of estrogen in bone cells` adaptive response to mechanical strain, we studied the effects of different estrogen concentrations on proliferation and differentiation of mechanically stimulated primary cultures of periosteum-derived osteoblasts.

Materials and Methods

Material

Cells were cultivated on polycarbonate plates (Nunclon, Wiesbaden/Germany). This material was chosen because it provides a good substrate for cell attachment, growth, and differentiation. The material can be bended without alteration of the integrity of the material or its surface structure. Peak surface strains of up to 20.000 μ strain can be reached. One strain means a 100% changing of the size.

Conjugated equine estradiol (CEE) was used as the hormonal agent. CEE was added in various concentrations to the medium (E_2 , Jenapharm, Jena/Germany). Medium concentrations of 0, 100 pmol, 10 nmol, and 10 μ mol E_2 were used.

Application of mechanical strain

It's proven that mechanical stimulation influences osteoblasts-like cells and bony tissue (Wiesmann, Neunzehn et al. 2009). In this study a four-point bending device was used to apply defined cyclic strains to the cells on the surface (Jones and Boyde 1976). Osteoblasts were grown for 24 h on the polycarbonate base plates in 12 cm² culture wells constructed of silicone rubber. Cultured cells were subjected to tensile uniaxial strains over the entire area of the base plates. Different strain rates were applied (0 and 3000 μ strains, respectively) at 1 Hz for a total of 200 cycles per day. This stimulation protocol was demonstrated in previous investigations to reveal an osteoblastic differentiation of cells. Cells were strained for 5 days. Investigations of proliferation and differentiation were performed two days after the final loading cycle.

Cell culture

Cells were prepared using the outgrowth method previously described by Jones and Boyde (Jones and Boyde 1976). The preparation led to a homogeneous culture of osteoblast-like cells, as indicated by the presence of osteocalcin and alkaline phosphatase. Briefly, periosteum pieces from radius and ulnae of 18-month-old steers obtained at the local slaughterhouse were cultured in High Growth Enhancement Medium (Flow Laboratories, Rickmansworth, U.K.) containing 10% fetal calf serum (FCS) for 4 to 5 weeks. Culture medium was changed once a week. After confluence, the cells were harvested by collagenase incubation (0.4 g collagenase and 98.8 mg Ham's F10 in 10 ml HEPES buffer) for 20 min followed by treatment with Tyrode's solution (300 mg ethylenediamine-tetraacetic acid (EDTA)-Na salt in 1000 ml solution, containing 200 mg KCl, 8 g NaCl, 1 g NaHCO₃, 50 mg NaH₂PO₄, and 1000 mg glucose). The cells were routinely tested for expression of osteocalcin and activity of alkaline phosphatase (16). Cell numbers were counted in a Cell counter. To examine the effect of calcium depletion, osteoblasts grown for 24 h on glass cover slips were exposed to EDTA solutions of different concentrations (0%, 0.005%, 0.01%, and 0.03%, respectively). After 20 min EDTA incubation in Tyrode's solution, cells were fixed with cold methanol (-20°C, 6 min) for indirect immunofluorescence assay.

Cell proliferation assay

Proliferation was measured by counting cell numbers 2 days after the last bending cycle. Osteoblasts were plated in Ham's F10 medium at a density of 1.5 x 10⁴ cells/cm². To minimize deviations of the

CEE concentration medium was changed every two days. Cells were harvested under the same conditions as described in the cell culture section; collagenase was incubated for 20 min followed by Tyrode's solution with a final pronase treatment (0.5 mg pronase/ml Tyrode's solution). The short pronase incubation was observed under phase-contrast microscopy until all adherent cells were detached. Cells were then counted in a Cell counter (Casy 1, TT, Schärfe System Germany).

Immunohistochemistry

For the detection of extracellular matrix synthesis cells were plated at a density of 6×10^4 cells/cm². Medium was then removed and plates were washed twice with PBS. Cells were then fixed in cold ethanol (-20°C) and air-dried. Monoclonal anti-osteocalcin, anti-osteonectin (Takara Biomedical, France), and anti-collagen type I (Bio Trend, Germany) antibodies were used in specific dilutions. Synthesis of proteoglycans was determined by anti-proteoglycan antibodies (Chemicon, Germany). AEC (3-Amino-9-ethylcarbazol) was used for the visualization of secondary antibodies (DakoEnVision, U.S.A.). Quantitative determination was performed in each group in 3 culture probes of 4 plates as described in detail by Hartig et al. (Hartig, Joos et al. 2000).

Statistics

Values are given as mean \pm standard deviation. Comparison between groups was performed by student's-t test. A p-value < 0.05 was considered significant.

Results and Discussion

Osteoblasts were viable during the whole culture period. They displayed the

phenotypic characteristics of osteoblast like cells. Cell morphology was more elongated during sub-confluency as seen for the proliferation determinations and changed towards a polygonal form when they reached confluency (as used for the evaluation of extracellular matrix synthesis) (Figure 1).

Proliferation of cells demonstrated statistically significant differences between control cultures and cultures exposed to estradiol after five day period (Figure 2). The number of cell decreased in mechanical stimulated as well as in non-stimulated cell cultures with an increasing estrogen concentration. At the highest estrogen concentration (10 μ mol) non-stimulated cultures even decreased while stimulated cultures show only a small proliferation.

The synthesis of extracellular matrix proteins of unstimulated cells revealed no statistically significant differences between different estrogen concentrations (Figure 3). The ratio of extracellular matrix proteins synthesis between stimulated and unstimulated cells was shown to be dependant on the estrogen concentration (Figure 4). High estrogen concentrations (10nmol, 10 μ mol) increased the effect of mechanical stimulation on the expression of bone associated proteins (osteocalcin, osteonectin) whereas no effects were present in control cultures or cultures with a low estrogen concentration (100pmol). The ratio of collagen type I and proteoglycan synthesis shows no statistically significant difference between mechanically stimulated and unstimulated cells by addition of estrogen (Figure 5).

A variety of cell culture systems have been employed to determine cellular effects towards estrogen. From these studies there

is extensive evidence that estrogen directly modulates osteoblast activity and indirectly regulates osteoclast formation and activity via the production of osteoblast-derived soluble and cell surface-associated cytokines (Spelsberg, Subramaniam et al. 1999). Modulation of osteoblast activity in turn influence bone function, imported also a respect to implant healing, when an undisturbed bone function is a prerequisite for a long term implant success. In general, it has proved difficult to demonstrate consistent effects of estrogen on primary, immortalized and transformed osteoblastic cells, particularly those of human origin. This can be explained in part by the generally low and variable levels of estrogen receptor (ER) in osteoblasts that may further diminish on culture, and by differences in cell source, methods of isolation, and culture. Resent research indicates that osteoblast – like cells express estrogen receptors at low level in culture (Odendal, Brady et al. 2004). It was observed that the expression of the ER is depended on the applied strain (Ehrlich, Noble et al. 2002) and the level of estrogen (Jessop, Suswillo et al. 2004). Despite these limitations we found that estrogen has a dose dependent influence on the osteoblasts` early differentiative response towards mechanical strain. It is evident from our results that estrogen has the ability to improve the synthesis of bone specific extracellular matrix proteins by osteoblasts in culture, whereas a significant decrease on cell proliferation was detected. Damien et al. (Damien, Price et al. 2000) demonstrated in contrast to our investigations a statistically significant increased proliferative response by 17 β estradiol on mechanically stimulated rat long bone – derived osteoblasts. They found that tamoxifen blocked the stimulating effect of estradiol

on the proliferative response of osteoblasts under mechanical loading. Their investigations revealed an estrogen receptor involvement in the adaptive proliferative response to mechanical strain. Our results indicate that estrogen has a stimulating effect concerning cell differentiation on mechanically loaded osteoblasts.

Withdrawal of the direct influence of estrogen on bone cells does not by itself explain the etiology of postmenopausal bone loss since its absence should be compensated for by the strain-related homeostatic mechanism(s) by which bone adjust their architecture to maintain target strains throughout their structure. It was recently demonstrated that also to its direct effects on bone cells estrogen influence their adaptive response to load bearing. The reduced capacity of the skeleton to maintain its structural competence following estrogen withdrawal could be therefore explained (Turner, Riggs et al. 1994). That estrogen receptor modulators block the proliferative responses to microstrain as demonstrated by our results suggests that the regulatory cascade by which strain stimulates mitogenesis also involves the estrogen receptor. If this reflects the situation in humans in vivo it could explain the diminished capacity of the postmenopausal skeleton to match its mass to its mechanical loading when estrogen is withdrawn and, among other changes, estrogen receptor activity may be down regulated. In reports to implant healing it is important to consider that data from the literature indicate that there is an effect of postmenopausal estrogen status on compromised implant healing in the maxilla. Typ I osteoporosis is associated with an accelerated loss of trabecular bone, sites with a disproportionate

Figure.1 Light microscopic image of osteoblast cell layer before mechanical stimulation.

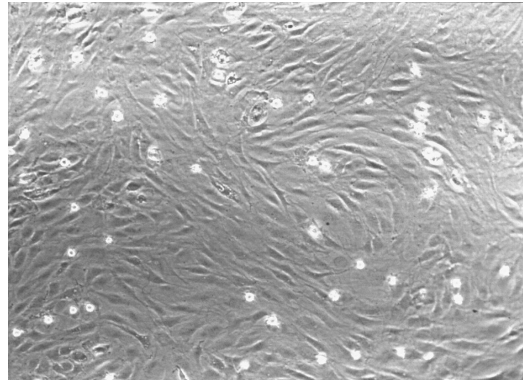


Figure.2 Cell proliferation of mechanically stimulated and unstimulated cultures in relation to different estrogen concentrations.

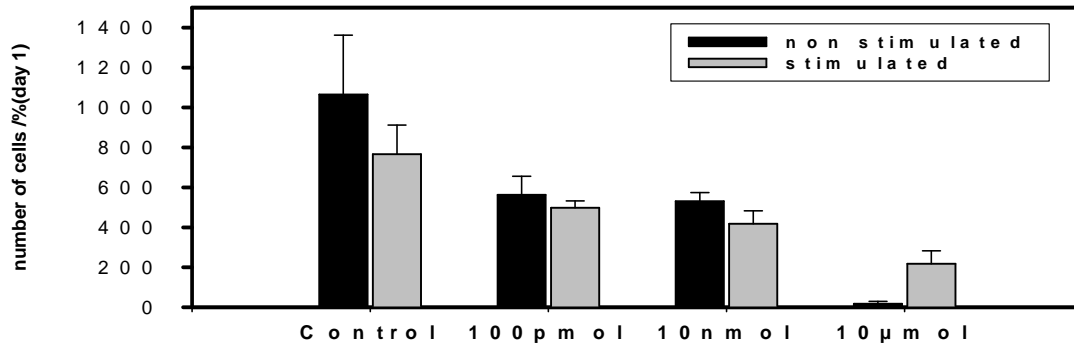


Figure.3 Staining intensity of mechanically unstimulated cultures in relation to different estrogen concentrations. Four bone-associated proteins (collagen type I, proteoglycan, osteonectin, osteocalcin) are shown. Values are given in % of the control (no addition of estrogen).

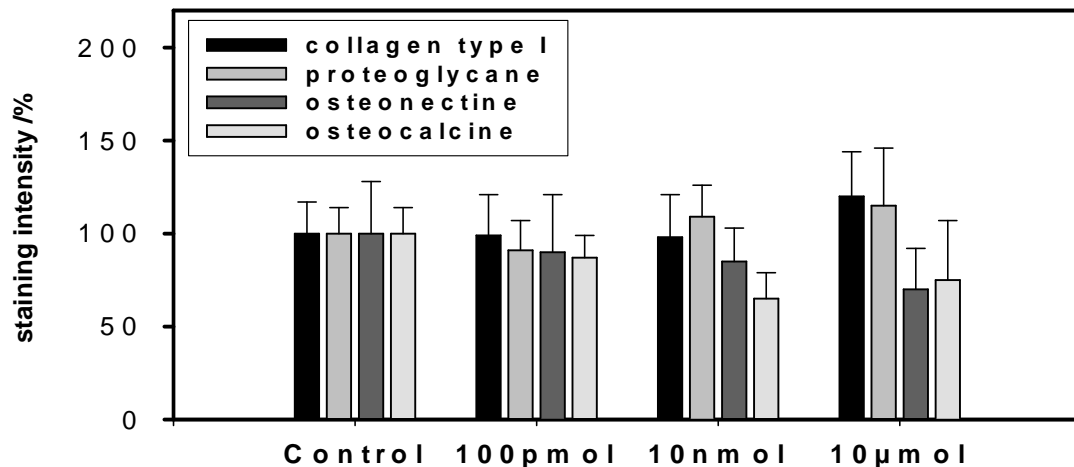


Figure.4 Ratio of the staining intensity of mechanically stimulated and unstimulated cultures for a) Osteocalcin and b) osteonection in relation to different estrogen concentrations. Values are given in %.

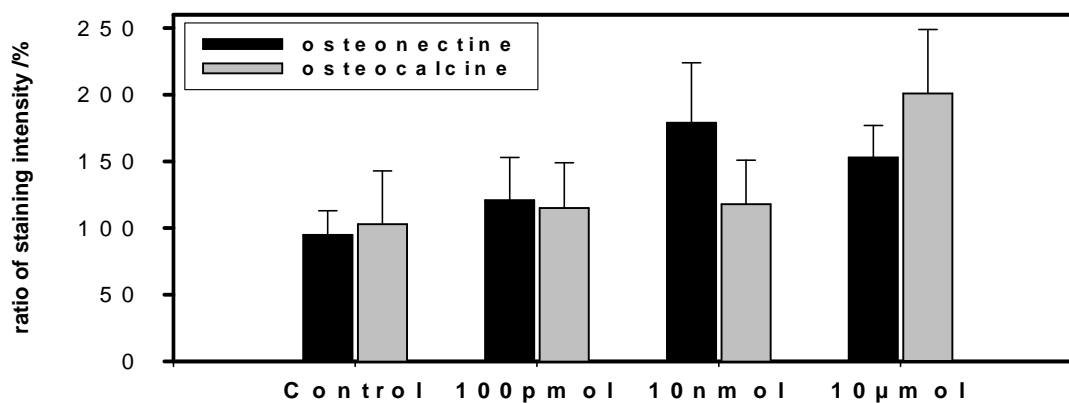
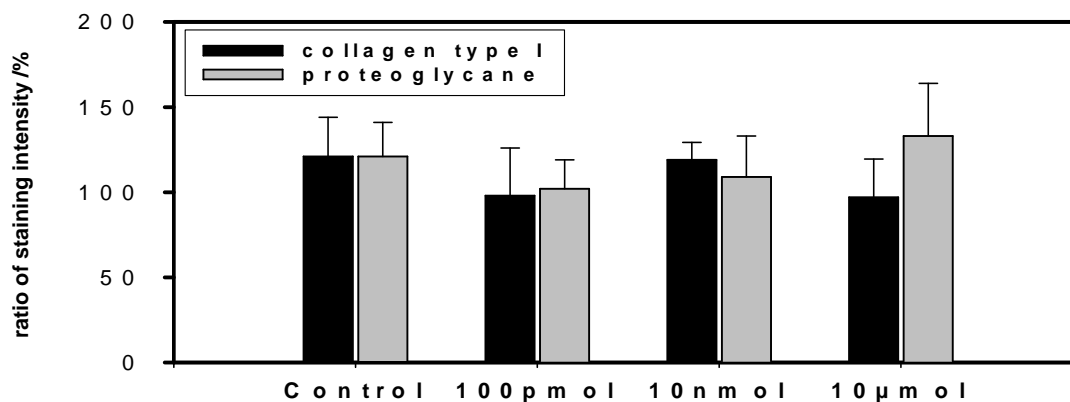


Figure.5 Ratio of the staining intensity of mechanically stimulated and unstimulated cultures for a) collagen type I and b) proteoglycans in relation to different estrogen concentrations. Values are given in %.



component of trabecular bone compared with cortical bone (maxilla) are at higher risk (August, Chung et al. 2001).

The reason for the clinical outcome of estrogen deficiency is not solved on a cellular level. Frost (Frost 1988) has suggested that estrogen deficiency shifts the "set point" for bone mass adaptation to mechanical loads in vivo. One of the factors determining the set point is the sensitivity of bone cells to mechanical stimuli. The hypothesis implies that, if

bone cells are more mechanosensitive, lower mechanical forces suffice to maintain skeletal bone mass. Under estrogen-deficient conditions, the response of bone cells to mechanical stimulation is reduced, leading to a net negative bone balance. Our in vitro results are in accord with this hypothesis, even if they relate only to the very early steps in the cascade of events leading to bone mass adaptation and maintenance. These findings on a cellular level may explain the finding that significant difference in rate of implant

failure was shown in the maxilla of postmenopausal women who had not received estrogen supplementation and in premenopausal women.

From a clinical point of view estrogen substitution by a hormone replacement therapy is therefore causative not only to protect bone loss by direct hormonal effects but also by an improvement of a load dependent bone gain.

Acknowledgement

The authors thank the lab teams of their groups and of the department for Cranio- and Maxillofacial Surgery of the university hospital Münster for the excellent technical assistance.

References

Akca, K., E. Sarac, et al. 2007. "Morphologic changes around biophysically-stimulated titanium implants in ovariectomized rats." *Head & face medicine* 3: 28.

August, M., K. Chung, et al. 2001. "Influence of estrogen status on endosseous implant osseointegration." *Journal of Oral and Maxillofacial Surgery* 5911: 1285-1289.

Bonnelye, E. and J. E. Aubin 2005. "Estrogen receptor-related receptor alpha: a mediator of estrogen response in bone." *The Journal of clinical endocrinology and metabolism* 905: 3115-3121.

Cadogan, J., A. Blumsohn, et al. 1998. "A longitudinal study of bone gain in pubertal girls: anthropometric and biochemical correlates." *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 1310: 1602-1612.

Damien, E., J. S. Price, et al. 2000. "Mechanical strain stimulates osteoblast proliferation through the estrogen receptor in males as well as females." *Journal of Bone and Mineral Research* 1511: 2169-2177.

Ehrlich, P. J., B. S. Noble, et al. 2002. "The effect of in vivo mechanical loading on estrogen receptor alpha expression in rat ulnar osteocytes." *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 179: 1646-1655.

Elsubeihi, E. S. and G. A. Zarb 2001. "Influence of estrogen status on endosseous implant osseointegration - Discussion." *Journal of Oral and Maxillofacial Surgery* 5911: 1290-1291.

Frost, H. M. 1988. "Vital biomechanics: proposed general concepts for skeletal adaptations to mechanical usage." *Calcified tissue international* 423: 145-156.

Gallagher, J. C. 2008. "Advances in bone biology and new treatments for bone loss." *Maturitas* 601: 65-69.

Hartig, M., U. Joos, et al. 2000. "Capacitively coupled electric fields accelerate proliferation of osteoblast-like primary cells and increase bone extracellular matrix formation in vitro." *European biophysics journal : EBJ* 297: 499-506.

Heilberg, I. P., E. Hernandez, et al. 2005. "Estrogen receptor ER gene polymorphism may predict the bone mineral density response to raloxifene in postmenopausal women on chronic hemodialysis." *Renal failure* 272: 155-161.

Higuchi, T., A. Tarakida, et al. 2009. "[Estrogens for treatment of osteoporosis]." *Nihon rinsho. Japanese journal of clinical medicine* 675: 954-

- 959.
- Jessop, H. L., R. F. L. Suswillo, et al. 2004. "Osteoblast-like cells from estrogen receptor alpha knockout mice have deficient responses to mechanical strain." *Journal of Bone and Mineral Research* 196: 938-946.
- Jones, S. J. and A. Boyde 1976. "Morphological changes of osteoblasts in vitro." *Cell and tissue research* 1661: 101-107.
- Odendal, M., K. Brady, et al. 2004. "The localization of estrogen receptors on human osteoblasts: a preliminary optical and electron microscopic technique." *Cell biochemistry and biophysics* 403: 237-247.
- Orbak, Z., H. Yildirim, et al. 1998. "Puberty and mineralization of the skeleton in girls." *Journal of pediatric endocrinology & metabolism : JPEM* 113: 421-425.
- Reid, I. R. 2008. "Anti-resorptive therapies for osteoporosis." *Seminars in cell & developmental biology* 195: 473-478.
- Riggs, B. L., S. Khosla, et al. 1998. "A unitary model for involutional osteoporosis: Estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men." *Journal of Bone and Mineral Research* 135: 763-773.
- Schiessl, H., H. M. Frost, et al. 1998. "Estrogen and bone-muscle strength and mass relationships." *Bone* 221: 1-6.
- Schlegel, W., L. I. Petersdorf, et al. 1999. "The effects of six months of treatment with a low-dose of conjugated oestrogens in menopausal women." *Clinical Endocrinology* 515: 643-651.
- Spelsberg, T. C., M. Subramaniam, et al. 1999. "The actions and interactions of sex steroids and growth factors/cytokines on the skeleton." *Molecular endocrinology* 136: 819-828.
- Turner, R. T., B. L. Riggs, et al. 1994. "Skeletal Effects of Estrogen." *Endocrine Reviews* 153: 275-300.
- Vanderschueren, D., J. Gaytant, et al. 2008. "Androgens and bone." *Current opinion in endocrinology, diabetes, and obesity* 153: 250-254.
- Wiesmann, H., J. Neunzehn, et al. 2009. *Biophysical stimulation of cells and tissue in bioreactors. Fundamentals of Tissue Engineering and Regenerative Medicine.* U. Meyer, T. Meyer, J. Handschel and H. P. Wiesmann. Heidelberg, New York, Springer: 633-647.