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Estrogen- Growth Hormone interaction in bone cells of the osteogenic lineage: Growth Hormone anabolic activity on human osteoblasts and their mesenchymal precursors is modulated by 17β-estradiol through a post receptor mechanism

BIO 14

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To my Family who taught me to give my best, always

Give the best you have, and it will never be enough.
Give your best anyway.

Mother Teresa

PUBLICATIONS DERIVED FROM THE PRESENT PhD PROJECT.

PAPERS

- **17β-Estradiol positively modulates growth hormone signaling through the reduction of SOCS2 negative feedback in human osteoblasts.**
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ABSTRACTS

- **Anti-adipogenic effect of growth hormone on human mesenchymal stromal cells derived from bone: possible role of microRNAs**
  S. Bolamperti, E. Mrak, G. Moro, G. Fraschini, Rubinacci, F. Guidobono, I. Villa
  XII CONGRESSO NAZIONALE SIOMMMS (Società Italiana dell'Osteoporosi del Metabolismo Minerale e delle Malattie dello Scheletro)
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- **Exposure of mesenchymal stem cells to estrogen and growth hormone induces osteogenic commitment even in an adipogenic culture environment**
  S.Bolamperti, I.Villa, E. Mrak, A. Rubinacci, F. Guidobono
  ABCD meeting "Stem Cells, Development and Regenerative Medicine"
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- **17ß-Estradiol facilitatory action on GH signaling in human osteoblast-like cells occurs via modulation of SOCS protein**
  S. Bolamperti, I. Villa, E. Mrak, P. Sirtori, G. Fraschini, A. Rubinacci, F. Guidobono
  3rd Joint Meeting of the European Calcified Tissue Society and the International Bone and Mineral Society Conference, Athens, Greece 07-11 May 2011

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Abstract

Abstract

In aging the loss of bone mass goes along with a decline of 17β-estradiol (E2) and Growth Hormone (GH), which are known to be bone anabolic factors. Recent evidence demonstrated in several cell lines an interplay between E2 and GH at a post receptor level. I thus evaluated the possible cross-talk between these two hormones in human osteoblast cells (hOBs) in primary culture and in their mesenchymal precursors (hMSCs).

E2 (10^{-8}M) given 60 min before GH (5ng/ml) enhanced both GH intracellular pathway in hOBs and the transcription of the evaluated GH target genes involved in osteoblast activity and matrix deposition, osteopontin (OPN), bone sialoprotein (BSP) and insulin like growth factor 2 (IGF2). E2 effects occurred by decreasing the protein levels of SOCS2, one of the main GH signaling inhibitors, through an increase in SOCS2 ubiquitination and consequent degradation. This effect was blunted by pre-treating the cells with the proteasome inhibitor MG132 (5μM). Interestingly this effect did not involve an E2 mediated genomic activity as Actinomycin D (5μM) pre-treatment did not prevent E2 modulation of SOCS2 levels *(Bolamperti S. et al., 2013)*. Further experiments demonstrated that this short term effect on SOCS2 levels was maintained over time: after 3h of E2 treatment there was still a decrease in SOCS2 levels. Yet, at this time point, the effect occurred via an inhibition of the transcription of SOCS2 gene. The fact that E2 negative regulation of the GH inhibitor SOCS2 involves an initially rapid protein degradation maintained for a longer time by a decrease in its gene expression strengthens the importance and the physiological relevance of this modulation for osteoblast activity. I was therefore interested to investigate whether or not two SERMs often used in clinics, Tamoxifen (Tam) and Raloxifene (Ral), share the same effect on GH signaling as E2. Cells treated with Tam (10^{-10}M) or Ral (10^{-8}M) 60 min before GH showed a trend to increase STAT5 phosphorylation even though reaching statistical significance, despite an observed reduction of SOCS2 levels with the SERMs alone. After 3h treatment no modulation of SOCS2 transcription was detected with either Tam, or Ral. These data suggest that despite their general

estrogen agonistic properties on bone, none of the two drugs displayed the same features as E2.

The combined effect of E2 and GH was evaluated also in mesenchymal stem cells (MSCs) obtained from human bones. The cells were first tested for plastic adherence, for differentiation capability towards adipocytes or osteoblasts, and for the positivity to CD73, CD105, and CD90 following an NIH protocol. In these osteoblast precursors, pretreatment of E2 60 min before GH, increased STAT5 phosphorylation induced by GH and decreased SOCS2 levels, as shown in hOBs. Considering the lack of information about GH action in stromal precursors, we evaluated GH action in the isolated hMSCs, focusing on its possible role in both osteogenesis and adipogenesis. The results showed that long term GH treatment (5ng/ml, 14 days, 3 times/week) increased early osteogenic genes and prevented adipogenesis in the isolated hMSCs. Given the important role of microRNAs in MSC commitment to osteoblastogenesis or adipogenesis, I analized if GH was able to increase miR-22 and miR-29c, considered amongst the main regulators of osteoblastogenesis or decrease miR-204, which is a regulator of adipogenesis.

GH was able to upregulate the transcription of miR-22 and miR-29c, without affecting miR-204. E2 *per se* inhibited their transcription and, in the combined treatment with GH, E2 pre-treatment was able to inhibit the stimulatory effect of GH on miR22 and miR29c. In conclusion the study has shown a relevant hormone to hormone interaction; hence E2 can locally modulate GH activity potentiating its cellular signaling in osteoblasts and in their stromal precursors. In osteoblasts the increase in the activity of the GH signal transducers reflects an increase in the transcription of GH responsive genes involved in the regulation of the deposition and the turnover of minerals and in the control of osteoblasts and osteoclasts metabolism (Gehron Robey and Boskey, 2006). In mesenchymal stromal cells the E2 potentiating effect of GH signaling was not accompanied by a positive effect on miRNA expressions. This could be due to the strong inhibitory effect that E2 *per se* exerts on the

evaluated miRNAs. Thus it can be suggested that the combined treatment of hMSCs with E2 and GH involves different mechanisms of the two hormones on microRNAs.

The data from the present study suggest that circulating estrogen levels should be considered in the management of GH replacement therapy in GH deficiency. Since the E2-GH cross talk is not shared by SERMs, the positive effect of E2 on GH signaling should be taken into account while developing new estrogen receptor modulators molecules.

1. Introduction

1.1 BONE TISSUE AND BONES

"Formation of the skeletal system is one of the hallmarks that distinguish vertebrates from invertebrates. In higher vertebrates the skeleton contains cartilage and mainly bone" (Yang, 2013).
Bone tissue comprises the largest portion of the body's connective tissue mass, but unlike other connective tissues, bone matrix (mineral, collagen, water, non-collagenous proteins, and lipids) is physiologically mineralized and has the unique property of being constantly regenerated throughout life by specific cell-types (Boskey & Gehron, 2013).

Considering its composition, bone tissue can be divided in woven bone or lamellar bone (Fig 1.1.1). The former is immature bone, non-mineralized, constituted only by collagen fibers and therefore very elastic, present during embryonic development, or soon after fractures. The latter is instead richer in mineral content and usually replaces the woven bone. Lamellar bone appears for the first time during the third trimester of gestation, and is filled with many collagen fibers parallel to other fibers in the same layer (lamellae) thus giving origin to several parallel fibers columns, forming the so called osteons. Osteons have in the middle the haversian canal, a tube around narrow channels in between the lamellae, able to contain up to two capillaries and nerve fibers. In cross-sections the lamellae run in opposite directions in alternating layers, a peculiarity that allows bone to resist torsion forces. In the lamellar bone it is possible to distinguish the compact or cortical bone and the trabecular or cancellous bone. The two differ for the tridimensional orientation of the lamellae, but not for the composition. Generally the hard outer layer of bones is composed of cortical bone with few gaps inside with porosity around 5–30%. The cortical compartment gives bones their smooth, white, and solid appearance, and accounts for the 80% of the total bone mass of the adult skeleton. The interior of the bone is filled of trabecular tissue, composed of a porous network of lamellae (porosity around 30–90%), that due to its structure,

allows room for blood vessels and marrow. Trabecular bone accounts for the remaining 20% of the total bone mass but has nearly ten times the surface area of the compact bone.

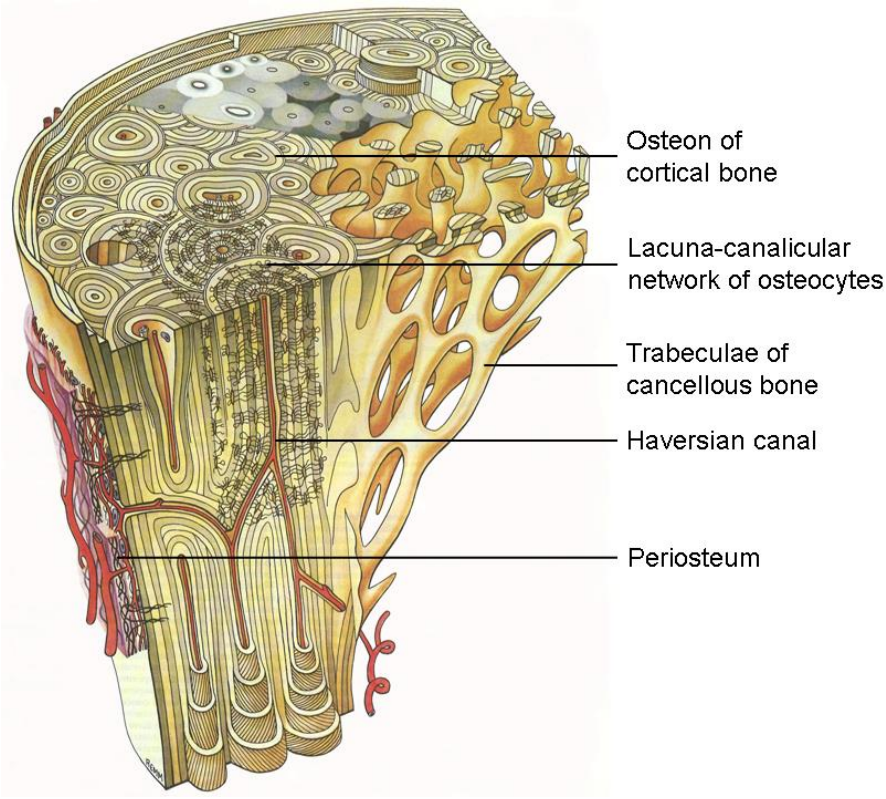


Fig.1.1.1 Schematic image of a cross-section through a layer of cortical and cancellous bone. Adapted from Gray 1918

In humans the adult skeleton is composed of 206 bones, which are covered outside and inside by two connective tissue membranes called *periosteum* and *endosteum*, respectively. The periosteum allows the longitudinal growth and protects the inner bone structure from traumas, whereas the endosteum,which is composed of a single layer of osteoprogenitor cells, is deputed to bone formation. Differing in shape, size, and functions, bones are divided in long,

short and flat bones. Long bones are longer than wider and are composed by a shaft called diaphysis and by two far ends, the epiphyses. These bones are almost made of cortical and trabecular bone. The diaphysis cavity (medullary cavity) contains the bone marrow. Short bones have only a thin layer of compact bone surrounding a cancellous interior and they are cube-shaped (e.g. wrist and ankle). The flat bones are thinner than the short ones and slightly curved. They are made of two parallel layers of cortical bone and with a a layer of trabecular bone between them (e.g. skull and sternum).

Besides the well-known properties of protection of internal organs, hematopoiesis, physical support of the motor apparatus, bones are endocrine organs deeply involved in the regulation and control of the whole body metabolism. They produce indeed body growth factors (i.e. IGFs, BMPs, TGFs), are fat and mineral storages, as well as the controllers of calcium and phosphate metabolism and the deputies to buffer the blood against excessive changes in the physiological range of pH.

1.2 BONE CELLS

The cells developing the various bone structures are the bone-resorbing *osteoclasts*, responsible of the digestion of bone matrix with the consequent release of minerals and signals factors; the *mesenchymal stem/stromal cells*, responsible for the expansion and healing of the skeletal tissue; the bone-forming *osteoblasts*, responsible for matrix production and mineralization, which can either become *osteocytes* once embedded in their own-made matrix, or undergo apoptosis or quiescence becoming *bone lining cells*.

OSTEOCLASTS

Osteoclasts are multi-nucleated cells with a diameter comprised between 20 and 200 micron. They derive from the hematopoietic stem cells and macrophage lineage precursors.

Osteoclastogenesis is driven by two essential cytokines: RANKL (Receptor Activator for Nuclear Factor κ B Ligand) and M-CSF (Macrophage-colony stimulating factor) (Boyle et al., 2003; Pixley et al., 2004). RANKL is member of the TNF family and it is necessary for the priming of osteoclasts precursors, whereas M-CSF contribute to the survival and proliferation. The production of these cytokines by osteoblasts, present either in soluble form or anchored to the matrix, is tightly regulated by several factors and it is dependent on the amount of osteoblast in the tissue. Upon stimuli as parathyroid hormone (PTH) or vitamin D (VITD) osteoblasts triggers osteoclastogenesis by secreting both M-CSF and RANKL or by exposing the latter on the membrane. Monocytes have the specific receptors, c-fms for M-CSF and RANK (Receptor Activator for Nuclear Factor κ B) for RANKL. Interestingly osteoblasts also secrete Osteoprotegerin (OPG, a specific inhibitor of RANKL which competes for RANK (Kostenuik et al., 2001). The balance between RANKL, and OPG is responsible

for osteoclastogenesis activation and consequently for bone turnover. Other cytokines responsible for osteoclasts development are IL-1, IL-2, IL-3, IL-6, IL-11, LIF (leukemia inhibitor factor) TNFs, oncostatin M, TGF-beta. Inhibitors are instead IL-4, IL-10, IL-18, INFγ and calcitonin, whose role is to induce retraction of mature osteoclast and quiescence.

Osteoclasts are often located in little lacunae (Howship lacunae) in which they secret acids and proteolytic enzymes able to digest both collagen and the inorganic matrix, solubilizing the minerals thus inducing bone resorption (Baron et al., 1989). The osteoclats activity develops in three different phases: adhesion to matrix, creation of a pH acid environment, digestion of the matrix.
The first step is mediated by integrins receptor. Once the cells have adhered to the matrix, they start protruding podosomes. A proton pump on the plasma membrane allows the acidification of the space within the podosomes ring, with the consequence of a local environment at pH 4.5, which is ideal for minerals solubility. The resorption process is physiologically relevant during bone repair and remodeling: a dysfunction can cause a severe human disease called osteopetrosis ,whose feature is the excess of bone (Teitelbaum et al., 2003).
A physiological bone turnover is necessary for the maintenance of bone mechano-properties. The turnover is usually activated either by micro-lesions or by homeostatic mineral needs. After the erosion of matrix by osteoclasts, osteoblasts migrate to the resorption site and start producing matrix to fill the Howship lacunae. As soon as the matrix reaches the right thickness it is mineralized by the deposition of calcium-phosphate crystals. All the cells involved in the whole process, both osteoclasts and osteoclasts, form a BMU (basic multicellular unit). These temporary bone anatomic structures, which derive from precursors resident in the bone marrow, completely disappear upon programmed apoptosis after the fulfillment of their duties. The process of bone resorption coupled with matrix apposition is the basis of bone

remodeling. In humans it covers a period up to 6-9 months (Parfitt et al., 1994).

MESENCHYMAL STEM/STROMAL CELLS

The Mesenchymal Stem Cells (MSCs) are the multipotent stem cells that give origin to the skeletal tissue. During development they form bone either by differentiating directly into osteoblasts in the intramembranous osteogenesis process or through endochondral osteogenesis in which they differentiate to chondrocytes with the formation of a cartilage template, ossified then by osteoblast (Yang 2013). In the adult skeleton MSCs give rise to several different cell types, as osteoblasts, chondrocytes, adipocytes, and myoblasts (Caplan et al., 1991, 2001; Pittinger et al., 1999)

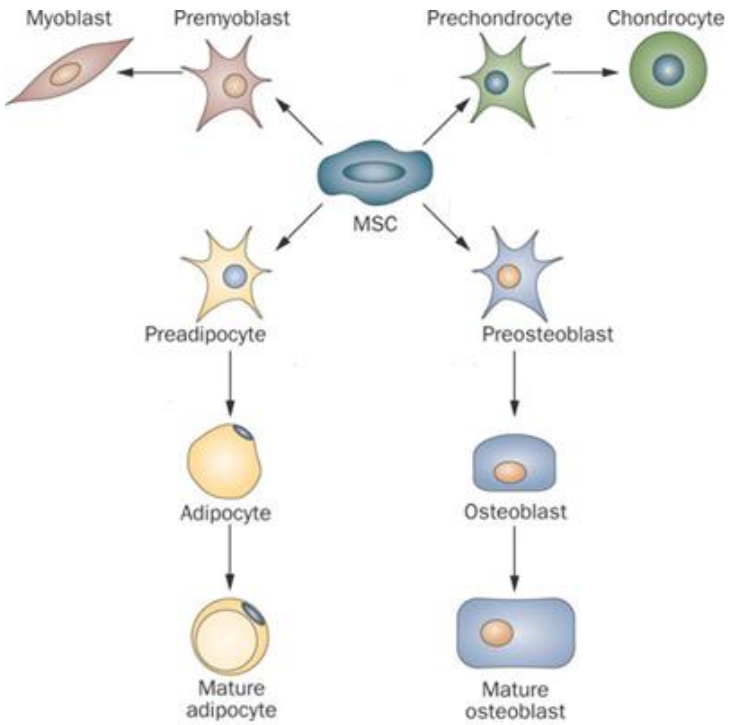


Fig 1.2.1 MSCs multi-lineage differentiation, modified from Takedaet al.(2009)

The osteogenic precursors were first reported by Friedenstein and colleagues in 1968 as *in vitro* spindle shaped, plastic-adherent, fibroblastic-like cells of the adult bone marrow, able to reconstitute *in vivo* the hematopoietic environment. In addition they showed that these cells could differentiate to bone and form colony forming unit (CFU-F) *in vitro* (Friedenstein et al., 1968, 1974). These experiments demonstrated for the first time the presence of a new cell type in the bone marrow stroma endowed with the potential of generating bone tissue. Based largely on these works, in 1988 it was postulated the existence of a stromal stem cell maintaining the marrow microenvironment as the hematopoietic stem cell maintains hematopoiesis (Owen &Friedenstein, 1988). However, convincing data to support the "stemness" were not forthcoming and it began a debate whether this MSC truly qualify a stem cell endowed of self-renewal properties and asymmetric division (Horwizt et al., 2000). Although a bona fide mesenchymal stem cell may reside within the adherent cell in the marrow compartment, the International Society for Cellular Therapy (ISCT) has recommended that these spindle-shaped, plastic-adherent cells derived from adult organisms should be termed "mesenchymal stromal cells" (avoiding the necessity to change the acronym MSCs) unless the capacity of self-renewal is proven *in vivo*(Hortwiz et al., 2005).

To better define their phenotype, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed three minimal criteria to define human MSCs. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105,CD73 and CD90, and lack expression of CD45, CD34, or in general of the hematopoietic markers. Third, MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006). Considering that the characterization of these cells is in constant evolution, nowadays it is under debate if the above criteria can still be considered valid, especially as regards the surface markers expression (Keating et al., 2012). However it is commonly accepted however that this population is very heterogeneous and only the 20-50% of plastic adherent cells represent multipotent MSCs, whereas

the remaining cells are a mixture of bipotential and unipotential cells (Muraglia et al., 2000).

Commitment of mesenchymal progenitors to different lineages is regulated by many hints coming from the local tissue microenvironment. It is known that there is an inverse reciprocal correlation between osteoblastogenesis and adipogenesis, with factors stimulating one of the two processes and inhibiting the other, resulting in increased osteoblastogenesis and decreased adipogenesis (Nuttal & Gimble, 2004).It has been proposed that bipotential osteoblast–adipocyte precursors form an intermediate in the process of commitment to these two lineages (Hasegawa et al., 2008). Two families of growth factors are at the moment the most studied due to their peculiarity to stimulate both osteoblastic and adipocytic differentiation: the Wnt (a portmanteau of Wingless and integration 1) family and the bone morphogenetic proteins (BMPs). As regards the Wnts Liu and colleagues demonstrated that they can favour osteogenic commitment in basal medium by inhibiting MSCs commitment into adipocytes, while inhibiting osteoblast differentiation in osteogenic conditions (Liu et al., 2009). Similarly BMPshave been shown to promote either adipogenesis (Sottile et al., 2000) or osteogenesis (Pereira et al., 2002) depending on the expression and availability of other differentiation factors. In addition the above described pathways GH, IGFs, estrogens, glucocorticoids, leptin are also involved in MSCs commitment.

PPAR-γ2 and the members of the CCAAT/enhancer binding protein (C/EBPs) are the major transcription factors activated during the adipogenic differentiation process. PPAR-γ2 is recognized as an essential transcriptional regulator of both adipocyte differentiation and lipid storage in mature adipocytes (Tontonoz et al., 2008), being its expression restricted only to this cell type (Bruedigam et al., 2008; Bruedigam et al., 2010). PPAR-γ2 overexpression in fibroblasts can stimulate adipogenesis (Tontonoz et al., 1994), whereas the KO results in the embryonic stem cells incapacity of

differentiation into adipocytes. Expression of PPAR-γ2 is regulated by C/EBP family of transcription factors, in particular by C/EBP-a. C/EBP is target of PPAR-γ2, thus creating a self-reinforcing regulatory loop (Tontonoz et al., 2008). C/EBPs can be induced by adipogenic hormones, like glucocorticoids and adiponectin. (The transcription factors and pathways involved in osteoblasts differentiation will be discussed in the following osteoblasts session).

A recent and new field of investigation concerns the role of microRNAs (miRNAs). These are short 18-25 nucleotides long non-coding RNAs, that regulate protein expression by binding to the 3-UTR of target messenger RNAs and promoting degradation of the target mRNAs or avoiding their translation (Erson et al., 2008). MiRNA expression patterns differ in MSC progenitors or fully differentiated cells, suggesting that miRNAs are highly relevant in MSC lineage decisions. Indeed, high or low expression levels of specific miRNAs may be a prerequisite for the commitment and differentiation of MSCs into specific lineages (Guo et al., 2011).
In human MSCs under osteogenic commitment miR-22 was found to be up-regulated and being a positive modulator (Huang et al., 2012). MiR-138 is instead inhibitory for osteoblastic differentiation (Eskildsen et al., 2008). MiR-204 (Huang et al., 2010) and miR- 143 (Esau et al., 2004) promotes adipogenic commitment.

OSTEOBLASTS

Osteoblasts are cube-shaped cells, juxtaposing one next to each other. Once closely connected they start producing bone matrix, forming the so called mineralization front. Osteoblasts features are synthetizing and secreting matrix components as collagen type I, vitamin K-dependent proteins, osteocalcin, osteonectin, bone sialoprotein, osteopontin (proteins fundamental for

anchoring cells during matrix deposition), proteoglycans, and alkaline phosphatase, the enzyme responsible for bone mineralization.

Osteoblasts maturation and functions are controlled by of specific stage-dependent transcription factors. The master regulator of MSCs differentiation towards the osteoblastic lineage is Runx releated protein 2 (RUNX2). RUNX2 is expressed in MSCs during skeletal fetal development and after birth it remains active during the whole osteoblast maturation (Karsenty et al., 2002). For these reasons its deficiency leads to the formation of a skeleton devoid of osteoblasts (Komori et al., 1997 Otto et al., 1997). RUNX2 was first found as a factor binding to an osteoblast specific cis-acting element in the promoter of Osteocalcin (Ducy et al., 1997), but consensus elements for RUNX2 have been also found in the promoters of many other oteoblastic genes, as osteopontin, bone sialoprotein, alkaline phosphatase and the chain I-α of collagen type 1. RUNX2 activity is however in mature/late stage osteoblasts inhibitory (Maruyama et al., 2007, Liu et al., 2001), suggesting that this factor is necessary for the maintenance of osteoblasts in a more immature stage (Komori et al., 2006). A second master regulator of osteoblast differentiation, downstream RUNX2, is osterix (Osx). Osx is a zinc finger transcription factor belonging to the SP family, specifically expressed in osteoblasts, and required for bone formation (Nakashima et al., 2002). Overexpression in MSCs results in an increase in alkaline phosphatase activity and osteocalcin expression with a parallel increase in newly formed bone (Tu et al., 2007; Wang et al., 2013). Other two transcription factors important in the osteoblast maturation process are ATF4 (activating transcription factor 4) and AP1 (activator protein 1). ATF4-deficiency decreased bone formation (Yang et al., 2004a), while forced accumulation of ATF4 induced osteoblastic genes expression even in non-osseous cells (Yang et al., 2004b). Its anabolic effect is fulfilled through the binding to RUNX2 favouring osteocalcin expression (Xiao et al., 2005).
The AP-1 proteins promote bone formation by binding physically to the promoters of several genes like osteocalcin, collagenase-3 (MMP13), bone

sialoprotein, and alkaline phosphatase, (Owen et al., 1990), regulating in this way their expression.

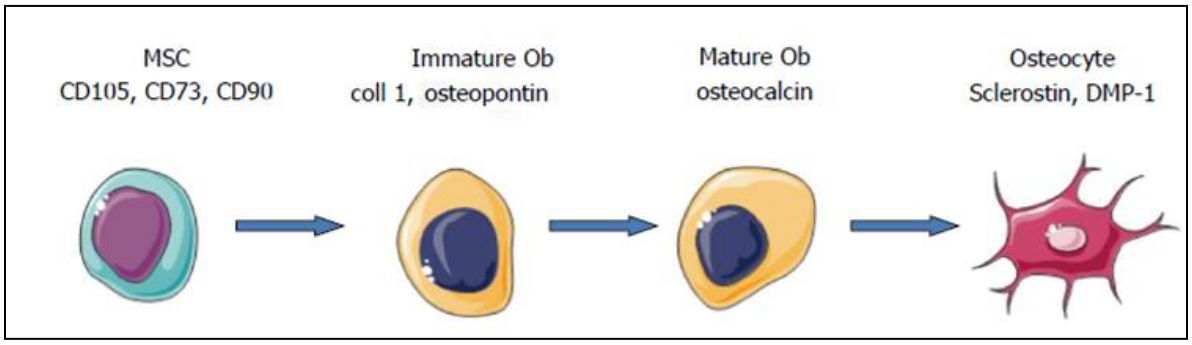


Fig 1.2.2 Osteogenesis, modified from Fakhry et al. (2013)

Factors that have a role in modulating the above described gene regulators are hormones like estrogens and growth hormone (that will be discussed further more in detail), BMPs (bone morphogetics proteins) TGF- β, FGF-2,vitamin D or parathyroid hormone (PTH). PTH can exert its anabolic effect on bone by activating simultaneously RUNX2 (Krishnan et al., 2003), Osx and inhibiting PPARγ (Wang et al., 2006) meanwhile being one of the principal activator of the AP1 complex.

Not only hormones are involved in osteoblast differentiation, but also mechanical loading and the canonical Wnt pathway, which acts via the activation of the transcriptional factor β-catenin. The canonical Wnt activation occurs upon binding of the secreted Wnt glycoproteins to both one of the seven-helix-receptors of the frizzled family and the co-receptorsLrp5 or Lrp6. At the steady-state β–catenin is usually phosphorylated and degraded. Upon Wnt stimulation or also PTH stimulation (Tobimatsu et al, 2006), β–catenin is released from the degradation complex and accumulates in the cytoplasm as unphosphorylated form. It then translocates into the nucleus where it binds the TCF/LEF complex and activates the transcription of bone anabolic genes, i.e. osteoprotegerin (OPG) or RUNX2.

β–catenin deletion in MSCs and early osteoblasts prevents the differentiation along the osteogenic lineage (Day et al., 2005) and two recent large genome-wide association studies identified Lrp5 as one of the highly significant genes associated with the bone mineral density (BMD) (Richards et al., 2008; van Meurs et al., 2008). Mutations in the Lrp5 domain responsible of the binding, to dickkopf1 (Dkk1), a major inhibitor of the pathway, are associated with high bone mass (Boyden et al., 2002), confirming the key role of Wnt in maintaining it.

Several microRNAs are involved in the osteogenic process. Among these worth of remark are miR-141, an inhibitor of osteoblasts differentiation and miR-210 which is known to increase the transcription of both Osx and ALP by inhibiting a repressor of osteoblast differentiation (Miyazono K et al., 2005). The osteogenic commitment is also promoted by the up-regulation of miR-29 family and is negatively affected when it is knocked down. Validated targets for miR-29 include several proteins that are inhibitors of osteoblast differentiation, such as histone deacetylase (HDAC)4 or TGFβ3 (Li et al., 2009). It was recently reported that canonical Wnt signaling rapidly induces the expression of both miR-29a and miR-29c (Kapinas et al., 2009).

After the maturation process one third of the osteoblasts become osteocytes, one third bone lining cells, whereas the remaining undergo apoptosis.

OSTEOCYTES

The matrix secretion by osteoblasts is usually polarized towards the surface and with regular intermittent interval towards other directions. In this way the osteoblasts take distance one from each other, being embedded in the matrix. At the end of this secretory phase the osteoblast starts slowing down its metabolic activity, sits in matrix cavities, called lacunae, and becomes an osteocyte.

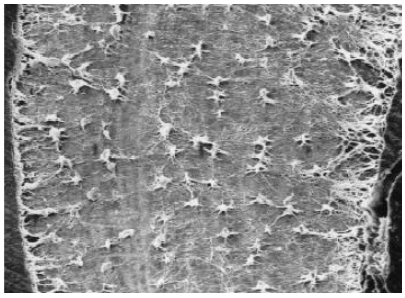


Fig 1.2.3 : SEM microphotograph (275X) at the middiaphyseal level of a metatarsal bone showing the methylmethacrylate casts of osteocyte lacunae and canaliculi. Periosteal and endosteal surfaces are to the left and right, respectivel (Rubinacci et al., 1998)

Osteocytes represents around the 90% of the cells present in bone. They are characterized by the presence on the outer membrane of long dendrites, which allow the cells to take nutrients from the circulation as well as releasing their secreted factors by going through the bone canaliculi. The factors released by dendrites are mainly matrix molecules or signals regulating bone and calcium homeostasis. At the far ends of the dendrites there are several gap junctions necessary for cell-cell communication between oteocytes and osteocytes-osteoblats. A feature of osteocytes is that they are the body mechano-sensors, able to transduce the mechanical stimulus into an electric or biochemical signal (Rubinacci et al., 2003). In the presence of a micro-lesion they can activate bone remodeling by secreting RANKL (Mulcahy et al., 2011) and they can regulate osteoblast activity and maturation through the production and secretion of a soluble inhibitor of Wnt pathway, sclerostin (SOST). SOST by binding to LRP5/6 prevents the activation of the intracellular signal (Burgers and Williams, 2013)

In humans osteocytes live many years, also decades. In bone of the elderly it is possible to find some empty lacunae, marker of osteocytes death, suggesting that with aging they undergo programmed apoptosis.

BONE LINING CELLS

Once the maturation process ends some osteoblasts stop their activity, reduce the volume of their organelles and transform from cube-shaped cells to long flat cells, the so called bone lining cells. Not much is known about their physiological role. It is generally thought that they mediate communication between the various bone cells and blood vessels. Recently it has been shown that bone lining cells have the capacity of de-differentiate into osteoblast upon PTH stimulation (Kim et al., 2012)

1.3 OSTEOPOROSIS AND AGING

More than 10 years ago, in 2000, around 200 million people were estimated being affected of osteoporosis (Dennison et al., 2000).
Osteoporosis is a chronic skeletal disease, predominantly of aging, which affects particularly postmenopausal women but also elder men. Aging process is generally characterized by a gradually deterioration over time of homeostatic processes, leading to functional decline and increased risk for disease and death. Metabolically, aging is characterized by insulin resistance, and consequent changes in body composition; physiological declines in growth hormone (GH), insulin-like growth factor-1 (IGF-1), and sex steroids, and by an increase in the metabolic waste, the reactive oxygen species (ROS) (Barzilai et al., 2012).
The economic burdens related to osteoporosis/osteoporotic fractures are significant. In the United States, based on the data from 2005, osteoporosis health care costs were c.a.17 billion dollars. These expenses are expected to increase by around 50% by 2025 (Komm et al., 2012).

The pathogenesis of osteoporosis is as complex as the network of diverse stimuli determining the strength of the skeleton (Rosen &Raid, 2013). Commonly it is defined as low bone mineral density (decreased bone mass per volume unit of anatomical bone) associated with skeletal fractures secondary to minimal or no trauma, most often involving spine hip and forearm. The decrease in bone mineral density is the consequence of an unbalanced bone remodeling process, with higher bone resorption than bone formation. The microarchitecture of bone, especially the trabecular, is deeply impaired (Follet et al., 2011). As suggested by two epidemiological studies in both genders the decrease in bone mass starts soon after thirties and it is not correlated to changes in sex hormones levels, whose decline is later in life (Looker et al., 1998; Riggs et al., 2008).

Nevertheless in women, compared to men, the loss of trabecular bone at the spine quickly accelerates after the menopause, as does the rate of fractures at the wrist and hip (Khosla S et al, 2005); attesting the positive role of estrogens on skeletal homeostasis. Cortical bone begins to decline after the age of 50 in both sexes.

Strong evidence for a pivotal contribution of aging to fractures was highlighted already 20 years ago by the work of Hui et al. (Hui et al., 1988), showing that in subjects with the same BMD, but with 20-yr of difference in age, there is a 4-fold increase in fracture risk in the older. During aging it occurs for example a deterioration of matrix proteins independent of hormonal status. Collagen type I undergoes severe changes leading to a structural impairment, as the loss of cross-linking between the component chains (Bailey & Knott 1999), with a consequent weakness of bone structure.

Despite being a major negative determinant of aging and lifespan (Giorgio et al., 2007; Russell et al., 2007; Lu et al., 2008) ROS cannot be avoided if living in an oxygen rich environment. In the cell the main sources of ROS are the mitochondria respiratory chain process and the fatty acid oxidation. ROS production can be induced by inflammatory cytokines and growth factors as well as external stimuli coming from the environment, i.e. UV light, or ionizing radiation. The administration of antioxidants to patients negatively regulates bone resorption with a positive effect on bone mass (Sanders et al., 2007; Pasco et al., 2006). Human clinical studies found indeed a correlation between the increase in oxidative stress and the decrease in BMD (Sánchez-Rodríguez et al., 2007;Oh et al., 2007; Altindag et al., 2008;) confirming previous pre-clinical data showing the occurrence of osteoporotic fractures upon OS damage in mice models of premature aging (Tyneret al., 2002; De Boer et al., J 2002).

Considering the prospective of bone loss in aging, osteoporosis can be considered one of the many factors responsible for the compromised bone strength that predisposes to an increased risk of fracture in a more general and multifactorial fragility syndrome (Manolagas, 2010).

OSTEOPOROSIS TREATMENTS

The aim of osteoporosis treatments is the prevention of fractures. Since the bone remodeling is unbalanced with an increase in bone resorption, the risk of fractures can be reduced either by inhibiting the resorption or by stimulating the formation. Due to the limitation of the current treatments, the bone research field is focused on finding novel antiresorptives and anabolics therapeutics.

The current anabolic treatments are PTH, sclerostin antibodies (phase III clinical trials) and some selective estrogen receptor modulators, (SERMS). Daily injections of PTH stimulate immature osteoblasts to become bone-forming osteoblasts with an increase in matrix production. After 6-9 months this increase in osteoblasts drives a coupled increase in osteoclasts with a consequent loss in therapeutic effect (Rosen, 2013). SERMs application is usually restricted to specific pathological conditions, as osteoporotic women with a high risk of invasive breast cancer, due to the side effects on other apparatuses (Masimov et al., 2013).

The common antiresorptive drugs are bisphosphonates and RANKL antibody. Bisphosphonates bind the matrix and prevent osteoclast resorption and induce osteoclasts apoptosis (Drake at al., 2008) RANKL antibodies inhibit osteclastogenesis by sequestering RANKL.

Blocking bone resorption is crucial for preventing the worsening of osteoporosis. However, the usage of antiresorptive drugs as unique osteoporosis treatment can lead to the inhibition of the physiological bone turnover, thus affecting bone quality. Moreover preventing osteoclasts activity is not sufficient to restore a normal BMD.

1.4 GROWTH HORMONE

Growth hormone (GH) is a circulating hormone with a half-life of 20-30 min whose blood concentrations range between 1 and 5-10 ng/ml. It is secreted from the pituitary gland following circadian cycles and its secretion is regulated by GHRH (growth hormone releasing hormone) and somatostatin, which are produced by hypothalamus. The former by binding to its specific receptor on the pituitary gland, favours GH production activating the transcription factors PROP1 and PIT1 (Potapov et al., 2006); the latter inhibits GH release thus determining the temporary windows of its activity. One of the major stimuli for GHRH production is hypoglycemia, which can be triggered i.e. by insulin administration or fasting. Others are intense physical activity, fever, stress, histamine, thyroxin, and serotonin.

GH is produced throughout life, however over 30s its levels start decreasing of 1% each year (Hermann et al. 2001). It is secreted as a mixture of different molecular variants. The most frequent form is a polypeptide of 191 amino acids, characterized by the bending of a single chain to form two rings linked together by di-sulfuric bridges. Two molecules of GH can also dimerize. Dimers can be present both in blood and the pituitary gland (Lewis, 1984).The coding gene for GH is located on the 17thchromosome and is called GH1. It situated in a cluster with somatotropin A and B (CSH1 e CSH2),placental growth factor (gh2) and CSH related genes. They all derive from a common ancestral precursor after duplication events, with identity of around 95% (Chen et al., 1989). Despite the common structure (5 exons separated by 4 introns), GH1is the only one expressed in the pituitary gland, and the others are produced in the placenta. New-born from GH deficient mums have normal weight and length. (Rimoin et al.,1966). The responsiveness to the hormone starts gradually increasing after birth, with a parallel increase in GH receptor in peripheral tissues (Gluckman et al., 1983; Maes et al., 1983).

As regards to GH biological effects, GH administration to hypophysectomized rats positively modulates nitrogen, phosphate, magnesium and potassium

metabolisms, skeletal and soft tissues growth. Effects of growth hormone on body different tissue can so generally be summarized as anabolic.

GROWTH HORMONE AND BONE

Among the anabolic effects on the body, the one on the skeletal development is the most known. GH is responsible of increasing height during childhood and it is important to maintain bone mass and homeostasis in the adults (Giustina et al. 2008). Congenital GH deficiency in humans and laboratory animals results in decreased bone growth and osteopenia (Ohlsson et al., 1998)

In 1957 Salmon and Daughaday performed a key experiment in the field: they noticed that normal serum stimulated the incorporation of S^{14} in rat cartilage, whereas GH *per se* had no effect. These observations induced the authors to postulate that GH has no direct effects in stimulating growth processes *in vitro* and *in vivo*, but these should be mediated by something downstream GH, present in serum. They called these unknown factors somatomedin (Sms). The name was then changed in IGF1 and IGF2 (Insulin like growth factors), due to their insulin like properties (Blundell et al., 1978). During embryonic development IGFs are fundamental, but act in a GH independent manner (Woods et al., 1996), whereas in the post-natal life their action on bone longitudinal growth is always associated to GH (Isaksson et al., 1987). IGF1 and IGF2 represent ca the 90% of the low kiloDalton protein from serum and are produced by the liver upon GH stimulation. Once produced they are released in the circulation system, where they can also bind to specific IGF binding proteins called IGF-BP (Zapf et al., 1975; Schwander et al., 1983). The role of the IGF-BP is to regulate IGFs availability and to increase their plasma half-life from 10 min to 3-4 hours. IGFs role is to promote proliferation and cell differentiation. They stimulate osteoblasts activity, favouring bone mass increase (Froesch et al., 1976).

In 1982 the concept that GH mediates its effect only through IGFs was revised by Isaksson and colleagues who showed that in vivo local administration of human GH to hypophysectomized rats resulted in accelerated longitudinal bone growth. Following these experiments other groups demonstrated a direct effect of growth hormone in favouring osteoblasts proliferation (Slootveg et al 1988), due to the presence of the GH receptor (GHR) on the cells (Morales et al., 2000). The expression of GHR is regulated by GH through a IGF1-mediated down-regulation (Leung et al., 1996)

Another important role of GH is the modulation of the osteoblast-osteoclast coupling, which occur through the stimulation of the OPG production and secretion in the local environment (Mrak et al., 2007).

A recent field under investigation is the role of GH in modulating MSCs commitment since GHRs are expressed by bone marrow MSCs (Kassem et al., 1994). There are *in vivo* evidences that bone marrow adipocytes are increased in number and size in GH deficiency dwarf rats (Gevers et al., 2002) and that GH suppresses bone marrow lipid accumulation (Menagh et al., 2010). Similarly to GH, IGF1 levels inversely correlate with bone marrow adiposity both in animals (Rosen et al., 2004) and in humans (Bredella et al., 2011).

GROWTH HORMONE AND AGING

During aging there is a progressive decline in GH secretion. In elder men can be even 20 fold less than in younger men (Ryall et al. 2008). The diminished GH secretion is not dependent on the pituitary gland, but it is the result of still unknown changes in the hypothalamus with the consequence of decreased GHRH and increased somatostatin (Veldhuis et al., 1996). As GH declines, IGF1 production and IGF1 systemic release are reduced (Landin-Wilhelmsen et al. 1994). The impairment of the GH/IGF1 axis correlates with a loss of BMD (Bohannon 1997) and bone itself , while aging, becomes less responsive to IGF1(Kveiborg et al. 2000). Pffeilschifter and colleagues showed that to elicit a

normal response to IGF1 it is necessary to use higher doses of the hormone when stimulating osteoblasts derived from elder patients compared to osteoblasts derived from younger ones (Pfeilschifter et al. 1993). These studies suggest that low circulating IGF1 and negative modifications of the GH/IGF1 axismay play an important role in the age-related affection of the skeleton system (Perrini et al., 2010).

GROWTH HORMONE SIGNALLING

GH exerts its action by binding to its specific receptor (GHR) present on the cell membrane. GHR is free of any enzymatic activity as the other members of the family of cytokines receptor type 1. It is characterized by an extracellular domain for the binding of GH, a single chain transmembrane helix, and an intracellular domain composed of two different motives, box1 and box 2, which able to bind both Janus Kinase 2 (JAK2) and the SH2 (Src Homologus)-domain proteins (Brooks et al., 2007). The signal transduction (Fig. 1.4.1) starts when GH binds to the extracellular domain of its receptor. Through crystallographic experiments it was shown that each single GH molecule binds simultaneously two GHRs, in two asymmetric sites, probably causing the receptor dimerization (de Vos et al., 1992). However recent data support the hypothesis that the receptor is already present in membrane as a dimer(Gent et al., 2002; Brown et al., 2005). In accordance with Brown and colleagues, GH binds these pre-cast dimers causing a consequent rotation of the intracellular domain. Since each cytoplasmic tail binds two molecules of JAK2, the conformational change is needed to get them closer, thus allowing their cross-phosphorylation. The kinases are activated and can then phosphorylate the receptor, allowing the recruitment of SH2 or PTB (Phospho-tyrosine binding) proteins, and triggering the activation of three different pathways:

1. JAK/STAT
2. MAPKs
3. PI3K/AKT

1. JAK/STAT PATHWAY

This pathway is the main intracellular signaling activated by GH. Once JAK2s are phosphorylated, they can phosphorylate and activate the STATs (Signal transducers and activators of transcription) family of transcription factors (Herrington et al., 2000). STATs can in fact bind the phospho-residues on either JAK2 or GHR, thus allowing JAK2 molecules to exert their kinasic activity. Once phosphorylated STATs can dimerize and translocate into the nucleus allowing the transcription of target genes.

As regard to GH, only one member of the family, STAT5is the responsible for its specific signal transduction (Gebert CA et al., 1999). STAT5 mutations in humans are indeed linked to severe growth delays. There are STAT5 responsive elements in IGF1 promoter too, suggesting that the observed phenotype could also be dependent on an impairment inIGF1 production (Woelfle et al., 2003; Chia et al., 2006).

2. MAPKs PATHWAY

The scaffold protein Shc can bind the activated complex GHR-JAK2. This binding is necessary for its phosphorylation and recruitment of Grb2 and SOS proteins, responsible of Ras activation. Ras can then phosphorylate Raf, which activates ERK1 and 2, upstream ELK1, the transcription factors at the end of the MAPK cascade (Winston et al., 1992, 1995; Vanderkuur, 1995).

This pathway can however be activated also in a JAK2 independent manner, through the recruitment in membrane of the tyrosine kinase Src, able to bind the phospo-tyrosines of GHR, with the involvement of phospholipase D signaling, which ends with activation of ERK1-2 (Zhu et al., 2002).

3. PI3K/AKT PATHWAY

The mechanism by which GH activates phosphatidil-inositol-3 kinase (PI3K) is the phosphorylation of the insulin receptor substrate, IRS, with consequent activation of IGF1 and IGF2 signaling (Costoya et al., 1999).

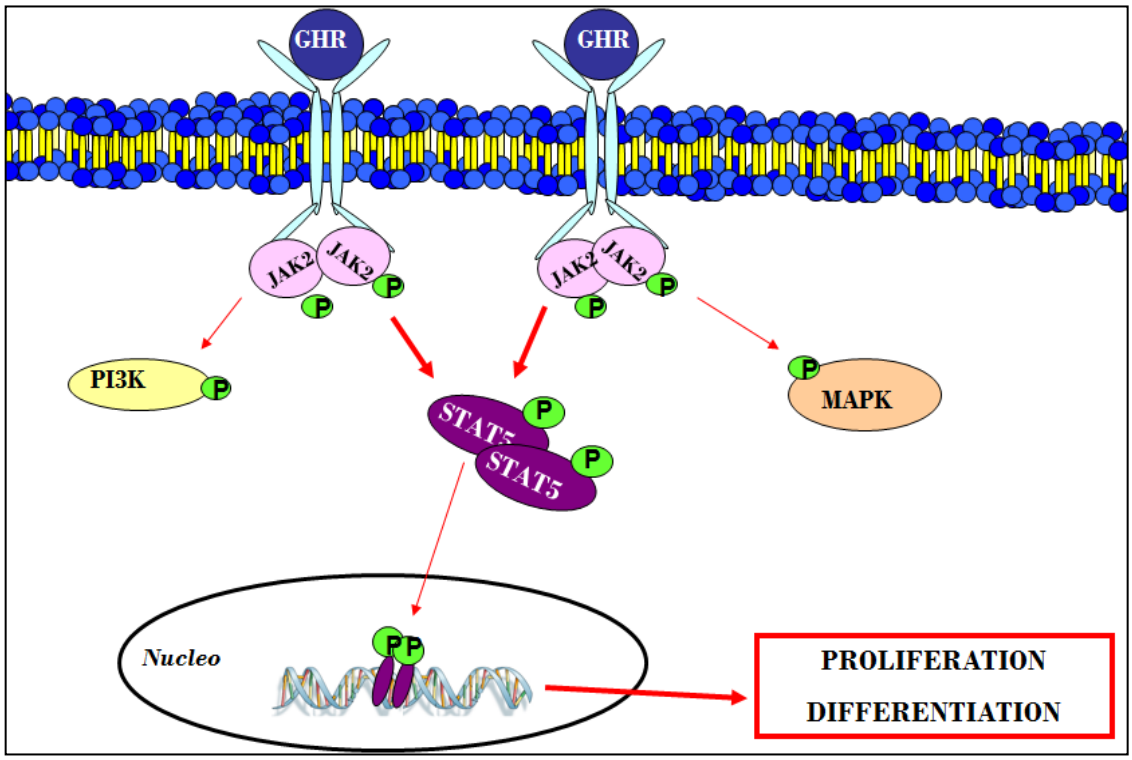


Fig 1.4.1 Schematic representation of the possible intracellular cascades activated by GH

The switching off of GH pathway is achieved by recruiting the suppressor of cytokine signals (SOCS) family.

Four members are involved in the negative regulation of the JAK/STAT pathway: SOCS1, SOCS2, SOCS3, and CIS (Flores-Morales et al., 2006). SOCS1 can bind the phospho-tyrosine of JAK2 in the catalytic domain,

inhibiting its activity (Yasukawa et al., 1999). SOCS3 can bind to both JAK2 and the phosphorylated residues on GHR (Hansen et al., 1999), whereas the precise mechanism by which SOCS2 regulates GH signaling is still unclear. Stronger evidences indicate that SOCS2 may bind both the SHP2-binding sites on the GHR and the phospho-tyrosine in the activation loop of JAK2, inhibiting JAK2 tyrosine phosphorylation itself and the activation of STAT5. Interestingly, SOCS2 actions may not be confined to regulating GH signaling. SOCS2 directly binds the IGF1 receptor and therefore it is possible that SOCS2 also regulates IGF-1 signaling, although IGF1 does not induce SOCS2 expression (Pass et al., 2009).

Another feature of SOCS proteins is that they are involved in the proteasome degradation of GHR due to the intrinsic activity of ubiquitin ligases (Flores-Morales et al., 2006). The receptor activity can also be blunted by its internalization or by the cutting off of the extracellular domain mediated by the Tumor necrosis factor α converting enzyme (TACE) (Zhang et al., 2000). The remaining domains are internalized and can translocate into the nucleus modulating gene transcription, common behavior with other similar hormones like ErbB (Linggi et al., 2006).

1.5 ESTROGENS AND 17β-ESTRADIOL

Estrogens are a group of steroid hormones, whose name derive from the greek words “oistros”, the name for women fertility period, and “gen” the suffix of the verb to generate. As the semantics indicate, these hormones are known for their role in developing the female phenotype.

There are 3 different biological estrogens: estrone (E1), 17β-estradiol (E2) and estriol (E3). In young non-pregnant ladies E2is the most prevalent; E1 is produced during menopause; E3 is the pregnancy hormone. In women estrogens are produced by the maturating ovarian follicle, by the placenta and the corpus luteum, whereas in men they are produced in the testes. Their synthesis is stimulated by FSH (follicle stimulating hormone) and LH (luteinizing hormone). Traces are also produced by liver, mammary glands, and adrenal gland. Estrogens derive from androgens metabolism. The synthesis starts by progesterone, which is converted in estrone or estradiol, directly or via the conversion of testosterone through the enzyme aromatase. Estrogens are important both in men and women. In men they are necessary for certain function of the reproductive apparatus and for sperm maturation (Hess et al., 1997; Raloff et al., 2008) and in women for the development of secondary sexual characteristics and the correct function of the menstrual cycle. In addition they regulate body fat distribution and favour triglycerides synthesis. Estrogens protect blood vessels from atherosclerosis by increasing the amount of HDL. Considering that overall women have more estrogens than men until menopause, they have fewer risks of cardiovascular diseases. Since estrogens are steroidal compounds, they generally diffuse through the lipid bilayer membrane and bind to the specific cytoplasmic receptor with the consequent activation/repression of gene transcription (Whitehead & Nussey, 2001).

E2 is the most potent among estrogens and is produced by the gonads, adipocytes and cerebral cells. For example the divergent females and males

behaviors can be ascribed to the levels of estradiol produced by the central nervous system during fetal development (Simerlyet al., 2002).

In the plasma E2 is usually bound to globulins or albumin, so that only a little fraction is free and biologically active. E2 levels are used as markers for follicle activity, menstrual dysfunctions, and menopause. High levels of E2often correlate with the presence of tumors.

E2 effects are not only restricted to the female reproductive apparatus. In liver it can inhibit IGF1 synthesis, in arteries it favours fluent blood flux (Collins et al., 2004), and loss of E2 in bone is linked to osteoporosis an osteopenia (Carani et al., 1997). Another major target organ for E2 is the brain, where it acts as a neuroprotective compound (Behl et al., 1995) and is involved in both pre- and postnatal brain development.

17β-ESTRADIOL AND BONE

E2 deficiency, caused both by menopause or ovariectomy, is associated with a rapid loss in bone mass, especially in the trabecular bone. Cortical bone loss is subsequent to the trabecular one and slower (Riggs et al., 2002). Even in males the lack of E2 affects bone mass; individuals carrying a deletion of the estrogen receptor α gene suffer from osteopenia and high resorption rate (Smith et al., 1994), the same phenotype is shared by males lacking the aromatase activity (Morishima et al., 1995)

Clinical studies have shown that E2 plays a role in inhibiting bone resorption. E2 inhibits RANKL expression, while increasing OPG (Eghbali-Fatourechi et al., 2003). Moreover it induces OCs apoptosis through the Fas pathway (Garcia AJ 2013). As regards osteoblasts it favours the maturation (Falahati et al., 2000) and inhibits the apoptosis (Bradford et al., 2010). It seems to also trigger the osteogenic differentiation of mesenchymal precursors. Dang and colleagues

showed using both murine primary cells and the cell line KS483, that E2 increases the osteogenic commitment and inhibits the adipogenic one, when cultured in osteogenic or adipogenic medium, respectively (Dang et al.,2002). The same results were shown using human MSCs (Zhao et al., 2011). However it seems that in MSCs not committed to any of the possible lineages, E2 exerts more a proliferative effect on the stromal precursors (Hong et al., 2011)

ESTROGENS AND AGING

The decrease of circulating estradiol during aging is associated to bone loss both in male and in female (Riggs BL 1998; Khosla S 1998; Tuck SP 2008).
In postmenopausal women the increase in the bone marrow adiposity (Syed et al., 2008) can be rescued by estrogen replacement therapy (Benayahu et al., 2000; Somjen et al., 2011). Moreover the anti-aging effects of estrogens include the protective effect against OS. Almeida and colleagues showed that estrogens have the capability of diminishing OS in bone and bone marrow, attenuating the OS induced osteoblasts apoptosis and the impaired osteoblastogenesis (Almeida et al., 2009).
Estrogen deficiency has also been associated with a reduced expression of SIRT1 (Elbaz et al., 2009), a NAD-dependent deacetylaserecently defined as a key longevity factor(Kim et al., 2012)

ESTROGEN SIGNALLING

E2 exerts its action through the binding to specific receptors, the estrogen receptors (ERs), which are mainly present in the cytoplasm. The ligand-receptor complexes dimerize and translocate into the nucleus where they bind to specific DNA palindromic regions called ERE (estrogen responsive elements, ERE= GGTCAnnnTGACC). Here they operate with co-activators or co-repressors of gene transcription (Klinge et al., 2000). They can also regulate the transcription of genes whose promoter is lacking the ERE consensus, by direct interaction with other transcription factors on the DNA, like AP1 or Sp-1(Safe et al., 2001). These complexes can also have a cytoplasmic activity independent of the DNA binding (Levin, et al., 2005).

Some ERs can be associated to the cell membrane and can be activated after exposure to the hormone. They are associated with G proteins, Tyrosine kinase (TK) receptors (i.e. EGRF, epidermal growth factor receptor) and non-TK receptors, like Src (Levin et al., 2005). Through the TK receptors E2 action is mediated by the activation of MAPKs and ERK pathway (Kato et al., 1995). E2 can also bind the GPR30 receptor (Otto et al., 2008), but the precise role of this receptor is still under investigation.

The first ER was cloned in 1986 by Green and colleagues (Green et al.,1986 a; Greene et al., 1986 b), and was considered the only onetill 1996. In that year it was indeed cloned the second E2 binding protein (Kuiper et al., 1996). The two receptors are nowadays known as ERα and ERβ. They have an identity of 97% in the amino acidic sequence of the DNA binding domain and only of 56% in the ligand binding domain. The genes coding for ERα and ERβ are ESR1 and ESR2 respectively. ESR1 is located on chromosome 6 whereas ESR2 on chromosome 14. They are present in almost all body tissues and usually in the same amount. In specific organs, however, one isoform can be more expressed than the other one. ERα is the most abundant in uterus, prostate stromal cells, ovaries, testes, breast, liver, white adipose tissue, some brain areas and bone. ERβ is mostly expressed in colon, prostate epithelium, vascular

endothelium, salivary glands (Karin et al., 2006). ERs are over-expressed in around the 70% of breast cancers and are considered to be the cause of the tumor formation. Indeed estrogens stimulate proliferation with an increase in DNA replication and a consequent increase in the risk of mutagenesis, and produce genotoxic waste through their own metabolism. In both cases the results is a misregulation of the cell cycle, of the apoptotic program and of the DNA repair. The involvement of ERα in breast cancer has been widely demonstrated, whereas the role of ER β is still under investigation. ERβ is however linked to the formation of the colon tumor (Harris et al., 2003)

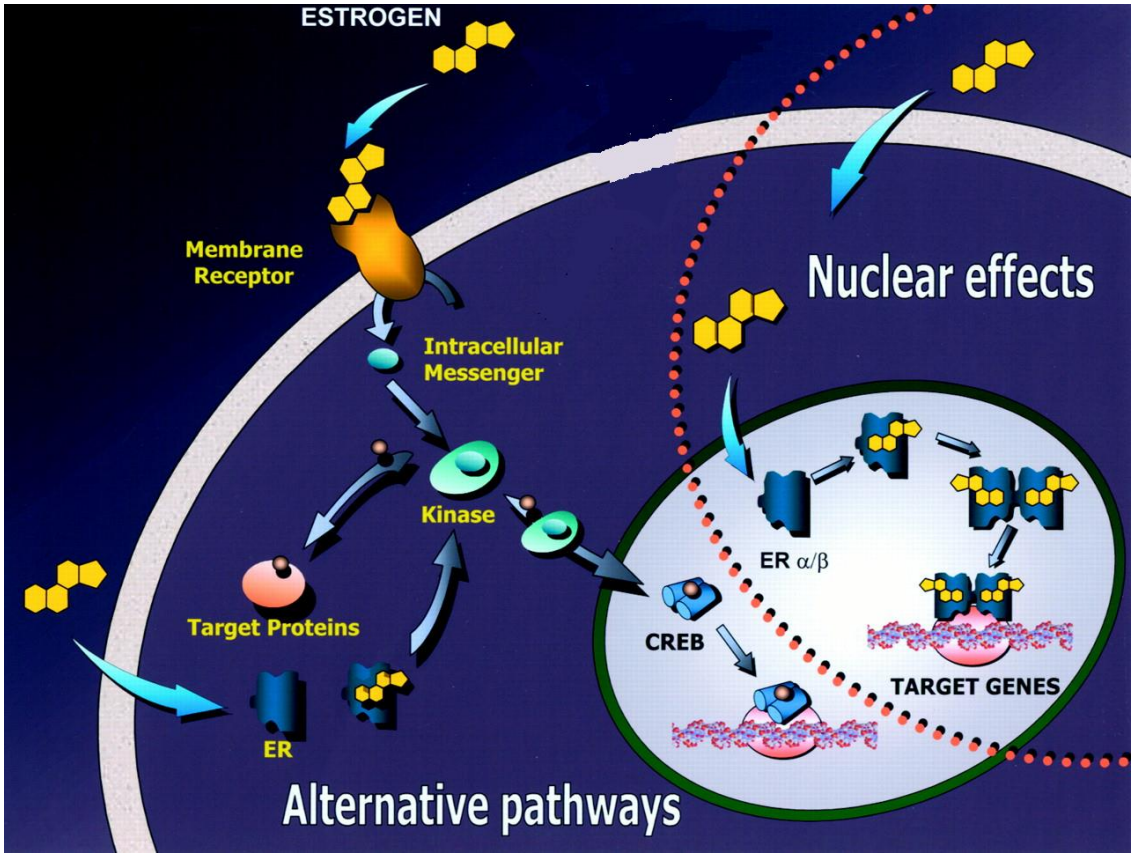


Fig 1.5.1 Schematic representation of the possible intracellular cascades activated by estrogens modified from Nadal et al 2001

1.6 SERMS

Selective estrogen receptor modulators (SERMs) are non-steroidal compounds binding the estrogen receptor.

SERMs display estrogen receptor agonist properties in some tissues and estrogen receptor antagonist in others (Goldstein et al., 2000). The molecular mechanisms responsible for the opposite responses to the same SERM are the different affinity for the receptor subtype and the diverse recruitment of co-activators/co-repressors in the cells (Dutertre &Smith 2000). SERMS molecules are studied since the 50s, although the very first public description of the possible use of SERMs in clinics was in 1987in New York, at the First International Chemoprevention meeting (Masimov et al., 2013). Among the SERMs molecules, Tamoxifen and Raloxifene, being among the first discovered, are the better characterized. They both have anti-estrogenic effect on breast and agonist properties on bone.

TAMOXIFEN

Tamoxifen has anti-estrogen activity in the breast and estrogen-like activity in the uterus (Fisher et al., 1994). The agonistic properties are driven by elevated intracellular levels of steroid receptor co-activator 1 (SRC-1), which are higher in the uterus than in the breast (Yatrik &Rowan 2005; Klessler et al., 2007).

In clinics tamoxifen use has been approved for the treatment of metastatic breast cancer, adjuvant therapy alone, for reducing the risks of cancer in high risk post-menopausal women, for men breast cancer and for the ductal carcinoma in situ treatment (Maximov et al., 2013). Clinical studies have highlighted cardio protective effects and bone anabolic properties (Love et al., 1992; Christodoulakos et al., 2006).

RALOXIFENE

Raloxifene has estrogenic actions on bone and anti-estrogenic actions on breast. Since raloxifene recruits mainly co-repressor proteins, it is an estrogen antagonist in the uterus, despite the high concentration of co-activators (Klesser et al., 2007). In 2006the National Cancer Institute announced that raloxifene was comparable to tamoxifen in reducing the incidence of breast cancer in postmenopausal women at high risk. One year later, the Food and Drug administration declared the raloxifene approval for the prevention and reduction of invasive breast cancer risks in postmenopausal women with high risks of osteoporosis, and for the treatment of osteoporosis in postmenopausal women with high risks of invasive breast cancer(Maximov et al., 2013). The trial for the Multiple Outcomes of Raloxifene Evaluation (MORE) found that postmenopausal women suffering from osteoporosis when treated 3 years with raloxifene 60 and 120 mg/day showed, compared with placebo, significantly reduced vertebral fracture risk by 30% and 50%, respectively (Ettinger et al., 1999). As well as tamoxifen, raloxifene induces vasomotor side effects (Stovall et al., 2007, Cohen et al., 2000).

2. Aim of the study

Most of the therapies for the treatment of osteoporosis are based on the inhibition of bone resorption and only few available therapies are anabolic (i.e. able to stimulate bone formation). Blocking the fast bone loss by inhibiting bone resorption is crucial for preventing the worsening of the disease. However, this therapy is not optimal because the inhibition of the physiological bone turnover could affect the bone quality. The blockage of osteoclasts activity might not be sufficient to restore a normal BMD. Hence characterizing the complex physiology of bone mass maintenance is the base to discover novel anabolic drugs for increasing BMD and decreasing bone fragility in the treatment of osteoporosis.

The GH/IGFs pathway is a complex process that regulates key aspects of growth and metabolism. GH is responsible of increased height during childhood and it is important to maintain bone mass and homeostasis in the adult (Giustina et al. 2008). Congenital GH deficiency in humans and animals results in decreased bone growth and osteopenia (Ohlsson et al., 1998). Estrogens are responsible for the development of secondary sexual characteristics, and they play a major role by regulating both somatic growth and the preservation of bone mass (Turner et al., 1994). There is a close interplay between estrogens and GH. The regulatory activity by E2 on GH may occur at many levels, secretion, clearance and action, and in different tissues. E2 favours the pituitary secretion of GH and can also influence the general responsiveness to GH replacement therapy in GH-deficient adults [Drake WM, 2001, Rossini A 2011]. In accordance to this evidence, orally administered estrogens reduce the metabolic action of GH in liver. In 2003 Leung and colleagues demonstrated that, in hepatoma and breast cancer cells, E2 inhibits GH-dependent activation of GH intracellular pathway (JAK2/STAT5) by increasing the expression of the inhibitor SOCS2 (Leung 2003). Interactions between hormones at a cellular level often occur in the organism (Birzniece et al., 2009) and post receptor modulations of cellular signaling could have a physiological relevance for reciprocal modulation of the activity.

In bone little is known about the possible regulation exerted by E2 on GH intracellular pathway. In the present study I decided to investigate the local interplay between E2 and GH that might occur in bone. For this purpose I evaluated the effects of GH and E2 in primary culture of human osteoblasts (hOBS) or their precursors, human mesenchymal stromal cells (hMSCs). Considering the widespread use of selective estrogen receptor modulators (SERMs) in therapy, I investigated if Raloxifene and Tamoxifen could display the same features as E2 in hOBs.

3. Materials and methods

3.1 DRUGS

Human recombinant growth hormone (GH), 17β-estradiol (E2), actinomycin D, MG132, Tamoxifen and Raloxifene were purchased from Sigma (Milan, Italy).

3.2 CELLS CULTURES

Human osteoblasts (hOBs) cultures

Human bone cell cultures were established by means of a modified version of the Gehron-Robey and Termine procedure (Gehron-Robey&Termine 1985) using trabecular bone samples obtained from waste material of female patients during orthopaedic surgery for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy. None of the patients (aged 68–81yr) had any malignant bone diseases and all of them gave their written consent for the use of the waste material. The protocol was approved by the Institutional Ethical Committee. No significant trend related to donor age was observed in any of the effects studied. Briefly, the trabecular bone was cut into small pieces and thoroughly washed with commercial standardized Joklik's modified MEM (Sigma, Milan, Italy) serum-free medium, to remove non adherent marrow cells. The pieces were incubated with rotation at 37°C for 30 min with the same medium containing 0.5 mg/ml type IV collagenase (Sigma, Milan, Italy), and collagenase digestion was stopped by the addition of Iscove's modified medium (IMDM, Lonza, Walkersville, MD, USA) containing 10% foetal bovine serum (FBS, Euroclone, Milan, Italy). Between eight and ten pieces from each patient were then placed in 25cm^2 flasks and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml mycostatin, and 0.25μg/ml amphotericin B until confluence; the culture medium was changed every 2–3 days. At the first passage the culture medium was changed to Dulbecco's modified essential medium (DMEM, Sigma, Milan,

Italy) without phenol red with 10% FBS and antibiotics as IMDM. The cell population was tested for alkaline phosphatase and osteocalcin production after $1,25(OH)_2D_3$ 10^{-8}M to ensure that the cells were endowed with osteoblast characteristics. Cells were used at first passage to reduce the possibility of phenotype changes.

Human mesenchymal stromal cells (hMSCs) cultures

Human mesenchymal stromal cells were isolated from the trabecular bone samples as described above for hOBs. The protocol was approved by the Institutional Ethical Committee. No significant trend related to donor age was observed in any of the effects studied. In accordance with the procedure of Zhou and colleagues (Zhou et al., 2008) the samples were washed several times with PBS to remove the remaining marrow from the bone. The washing solution was centrifuged and the pellet resuspended in 10 ml of PBS. The mononucleated cells were isolated on a density gradient centrifugation (Histopaque-1.077, Sigma, Milan, Italy). The isolated cells were then plated in a 25 cm^2 flasks. After 24 hours, the medium was replaced with fresh one to eliminate the non-adherent cells. Cells used for FACS analysis and experiments were at the third/fourth passage as suggested by NIH (Horwitz 2006). hMSCs were cultivated in 10% FBS low glucose phenol red-free Dulbecco's modified essential medium (DMEM, Sigma, Milan, Italy) with antibiotics. For GH and E2 treatments cells were cultivated in DMEM supplemented with 1% FBS and 9% charcoal stripped FBS (Sigma, Milan, Italy). The GH and E2 concentrations were the same as for OBs.

3.3 FACS ANALYSIS

The expression of specific MSC markers, CD105, CD73, CD90, CD34, CD45 was evaluated by flow cytometry (FACS). A total amount of 10^5 harvested cells was used for each experimental condition. After washing with PBS-FACS (PBS + 0.1% BSA), the cells were incubated for 20 min with the following fluorescence-labeled antibodies diluted 1:200: CD90-APC (BD Pharmingen), CD73-PE (BD Pharmingen), CD105-biotinylated (R&D systems, MN, USA) CD34-PE-Cy5 (BD Pharmingen, NJ, USA) and CD45-Pacific Blue (BD Pharmingen, NJ, USA). Surface staining was followed by PE-Cy7-conjugated secondary antibody (BD Pharmingen) staining for the detection of CD105. To determine the background fluorescence for each antibody, both unstained samples and immunoglobulin-matched isotype samples were included in each analysis.

Samples were acquired immediately with an LSRII FACS (BD Pharmingen) and analyzed using FlowJo software 2.2 (TreeStar Inc., OR, USA). Forward scatter-area versus forward scatter-height properties were used to exclude cell aggregates, and live cells were separated from dead cells. The cells were first gated for CD90 and then for CD73 and CD105 expression. In the triple positive population, the frequency of CD45 and CD34 double positive cells was calculated.

3.4 DIFFERENTIATION ASSAY

Differentiation assays were performed on hMSCs challenged for 14 days with pro-osteogenic medium (osteoMEM) that consisted of growthMEM supplemented with 50 µg/ml 2-phosphoascorbic acid, 10 mM ß-glycerophosphate, and 10^{-7} M dexamethasone, or with pro-adipogenic medium (adipoMEM) containinggrowthMEM supplemented with 5x10-4 M 3-isobutyl-1-methylxantin (IBMX), 10^{-4} M indomethacin, and 10-6 M dexamethasone. The controls consisted of cells cultured in growthMEM.

To assess osteogenic differentiation, cells were fixed in 70% ethanol in sterile water for 10 min and stained with Alizarin red S (40 mM, pH 4.1–4.3) for 20 min to color the mineralized matrix. Images were acquired using an Epson Scanner. To assess adipogenic differentiation, cells were fixed in 60% isopropanol for 10 min and then stained with Oil Red O (ORO) (0.3% w/v in 60% isopropanol) for 15 min to color the lipid droplets present in the newly-formed adipocytes. Images were taken with an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 10X or 20X magnification. The accumulation of ORO was quantified after de-staining of the cellular monolayer by incubating it in 100% isopropanol for 10 min and measuring the absorbance at 500 nm on a plate reader, Infinite 2000 (Tecan Italia s.r.l., Milan, Italy). Results were expressed as a ratio of the absorbance to the DNA content of each sample.

3.5 REAL TIME qPCR

Real Time PCR for messenger RNAs

Total RNA from confluent hOBsor hMSCs was extracted using TRIzol according to the manufacturer's instructions (Invitrogen Life Technology, Inc., Paisley, UK). RNA pellets were dissolved in sterile distilled water and their concentrations were assessed by spectrophotometric analysis (OD260/280). One µg of total RNA was retrotranscribed in a total volume of 25µl using an oligodT primer (0.5µM), 200 U of M-MLV Reverse Transcriptase, deoxynucleotides (0.5mM), M-MLV reaction Buffer 1x and rRNasin Ribonuclease Inhibitor 1U/µl (Promega Corporation, Madison, WI, USA).

The relative expression of osteopontin (OPN), bone sialoprotein (BSP), and IGF2 mRNA was evaluated in hOBs. At confluence after 24h of serum starvation, cells were treated for 6 h with GH (5 ng/ml) or E2 (10^{-8} M). For the combined treatment with the two hormones, E2 was added 60 min before GH. The relative expression of SOCS1, SOCS2 and SOCS3 mRNA was evaluated 60 min after treatment with E2 (10^{-8} M)/Tam(10^{-10} M)/Ral (10^{-8} M) In human MSCs the expression of OPG, OSX, RUNX2, ADN, C/EBP α was evaluated after 14 days long term treatment with GH (5ng/ml; 3 times/week).

Relative quantification of mRNA expression was performed on an ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA) using 10 ng cDNA of the RT-PCR solution in a final volume of 25µl. The primer-probe sets were purchased as Assay-on-Demand for gene expression from Applied Biosystems. Real Time PCR was performed with FAM labelled specific probes for IGF2, OPN, BSP, SOCS1, SOCS2, SOCS3, OSX, RUNX2, ADN, C/EBPS and β-actin, which is the housekeeping gene used as endogenous control.

Real Time PCR was run according to the following protocol: an initial step of 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15s at 95°C and 1 min at 60°C. mRNA levels were quantified using the comparative threshold-

cycle (Ct) method. First**,** the amount of target mRNA in each sample was normalized to the amount of the housekeeper mRNA (β-actin), designated as a calibrator, to give ΔCt (Ct_{target} - $Ct_{\text{-actin}}$). Second, the amounts of target mRNA in the samples were expressed using the formula: Amount of target mRNA = $2^{-\Delta\Delta Ct}$, where ΔΔCt = $\Delta Ct_{\text{(sample1)}}$ - $\Delta Ct_{\text{(untreated sample).}}$ Three replicates were performed for each experimental point and experiments were repeated several times with cells obtained from different donors.

Real Time PCR for microRNAs

In human MSCs the expression of miR-22, miR-29c and miR-204 was evaluated after 14 days of GH or E2 (5ng/ml, 10-8M respectively; 3 times/week); for the combined treatment cells were pretreated 60' with E2 before GH. For each RT reaction, in accordance with the manufacturer's protocol (TaqMan® Small RNA Assays, Applied Biosystems), 10 ng of RNA were combined with a RT master mix containing 1mM dNTPs, MultiScribe™ Reverse Transcriptase 50 U, 1X Reverse Transcription Buffer, RNase Inhibitor 3,8 U and 1X of the kit-included specific primers. The reaction was performed for 30 min at 16°C, thenfor 30 min at 42°C, with a final 5' step at 85°C. From this reaction 1.33 µl were used for the Real Time PCR amplification. The cDNA was mixed with 1x TaqMan® Small RNA Assay and 1X TaqMan® Universal PCR Master Mix II. The PCR reaction was performed following the same protocol used for the mRNAs.

3.6 WESTERN BLOT ANALYSIS

Phosphorylated-STAT5 (P-STAT5) and STAT5 protein levels were tested in confluent hOBs or hMSCs after 24h of serum starvation and treated for 60 min with different doses of GH (0.1ng/ml-20ng/ml), or E2 (10^{-8}M), or Tam(10^{-10} M), Ral (10^{-8} M) or E2/Ral/Tam with GH (5ng/ml). In this latter case the treatment was added 60 min before GH. SOCS1, SOCS2 and SOCS3 protein levels were detected in 24h serum starved hOBs after 60 min treatment with E2 (10^{-8} M) Tam(10^{-10} M)/Ral (10^{-8} M). Actinomycin D (5μM) was added 30 min before E2. After removing the medium, adherent cells were gently scraped into 75μl lysis buffer (Hepes 50mM, NaCl 250mM, $MgCl_2$ 5mM, 1% Triton, 10% Glycerol, protease and phosphatase inhibitors cocktail (1:100, Sigma, Milan, Italy). The lysates were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected and the total protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA). Thirty-five micrograms of total protein extract were mixed with the appropriate volume of Laemmli's sample loading buffer, heated at 100°C for 5 min and loaded onto 10% SDS polyacrylamide gels. Western blots were performed using specific antibodies against human P-STAT5, STAT5, SOCS2, SOCS1 and SOCS3 (Cell Signalling Technology, Boston, MA, USA) diluted 1:200, 1:1000, 1:500 and 1:1000, respectively, in 5% milk or BSA Tris Buffered Saline with 0.1% Tween20. After rinsing, membrane was treated with specific horseradish peroxidase conjugated secondary antibodies (1:2000) and enhanced chemiluminescence substrate (ECL, Pierce). Bands on x-ray films were then quantified using Scion Image software (Scion Corp., Frederick, MD, USA). β-actin was used as an endogenous control to avoid incorrect estimations of the signal of the protein tested. The intensity of the treated samples were normalized to that of the untreated samples (untr).

3.7 CO-IMMUNOPRECIPITATION

For the immunoprecipitation assays hOBs were treated 60 min with E2 (10^{-8}M) and lysed in RIPA Buffer added with protease and phosphatase inhibitor cocktail (Sigma, Milan, Italy). Five hundred micrograms of these total protein extracts were incubated for 2h at 4°C with 2µg of anti-ubiquitin primary antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany). Then 20 µl of Protein A-agarose (Santa Cruz Biotechnology Inc., Heidelberg, Germany) were added and samples incubated on a rotating device overnight at 4°C. The so formed sepharose bound immune complexes were collected by centrifugation at 4°C for 5 min at 3500 rpm. Pellets were washed 4 times with 1ml of PBS, each time repeating centrifugation step with the following conditions: 6 min at 3500 rpm; twice 6 min at 3000 rpm, 7 min at 3500 rpm. After the final wash pellets were resuspended in 40 µl of Laemmli sample buffer denaturating 1x and boiled for 3 min. Twenty microliters of each sample were used to perform a SDS-PAGE assay, followed by immunoblot analysis. The blots were quantified by Scion Image software as described for western blots.

3.8 siRNA-MEDIATED SOCS2 GENE SILENCING

Subconfluent (80%) hOBs were transfected with a pool of predesigned short interfering RNAs (FlexiTube GeneSolution for SOCS2, Qiagen, Milan, Italy) targeting human SOCS2 and a control scrambled RNA (AllStars Negative Control, Qiagen, Milan, Italy), targeting a sequence not sharing homology with the human genome using the RNAiFect transfection reagent (Qiagen, Milan, Italy) according to the manufaturer's instructions. hOBs were incubated with the transfection complexes for 48 hrs at 37°C and 5% CO_2 before treatment. A ratio of 1µg siRNA: 3µl RNAifect was used as it provided an average gene silencing of 75% as determined by real time PCR (Fig.4:8A).

3.9 STATISTICAL ANALYSIS

Statistical analysis was performed by means of the statistical package Prism vs 4.00, (GraphPad Software, San Diego, CA, USA). Statistically significant differences were determined using the nonparametric ANOVA test (Kruskal-Wallis test) followed by multiple-comparison test (Dunn's post-test) or by the Mann-Whitney test.

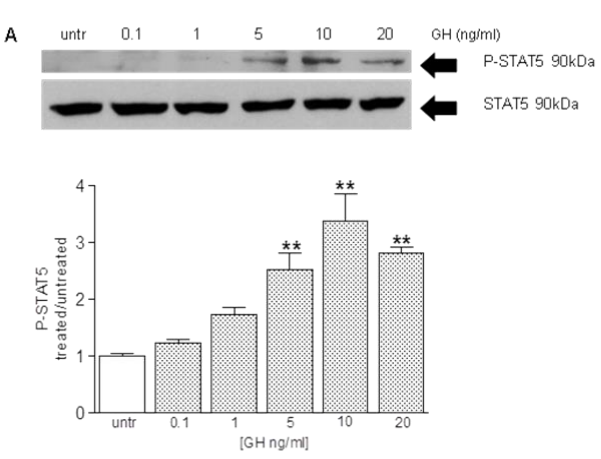
4. Results

4.1 STAT5 phosphorylation induced by GH and E2 in hOBs

Since JAK2/STAT5 cascade is the major pathway activated by GH, I first evaluated the concentration of GH and the time-course required to induce the activation of this pathway in hOBs. Different concentrations of GH (0.1, 1, 5, 10, and 20 ng/ml) induced a concentration-dependent increase in the phosphorylation of STAT5 (P-STAT5) after 60 min exposure. The increase in P-STAT5 was statistically significant from 5ng/ml ($P< 0.05$) to 20ng/ml ($P< 0.01$) (Fig. 4.1 A). GH treatment (5ng/ml) induced a time-dependent increase in P-STAT5 that became significant from 30 min until 90 min (Fig. 1B). Considering these results, I chose the following experimental conditions for the assessment of STAT5 phosphorylation: a GH concentration of 5ng/ml and 60 min treatment.

I then analysed E2 possible modulation of GH intracellular pathway. E2 pre-treatment (10^{-8} M) 60 min before GH induced a significant increase ($P<0.01$) of P-STAT5 compared with GH alone, E2 (10^{-8}M) *per se* had no effect on P-STAT levels. In hOBs treated with the two hormones at the same time, E2 did not increase GH dependent STAT5 phosphorylation (Fig. 4.2). The total amount of STAT5 did not change after exposure to either GH (5 ng/ml) or E2 (10^{-8}M) (Fig. 4.1-2). On the basis of these results cells were always pre-treated with E2 60 min before GH.

Fig. 4.1



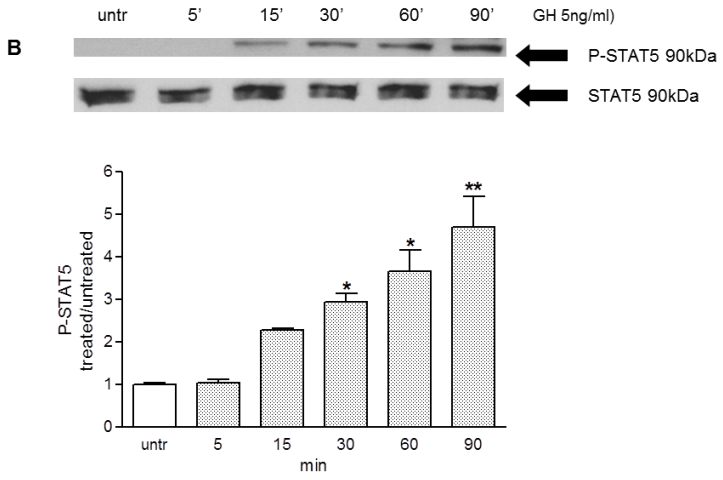


Fig. 4.1

Fig. 4.1.A: *STAT5 phosphorylation (P-STAT5) in responseto increasing concentrations of GH (0.1-20 ng/ml) after 60 min treatment in hOBs ; representative Western blots and relevant quantification, as described in the Materials and Methods (n = 6). No changes in the total amounts of STAT5 were observed* ***B:*** *Time-course of 5 ng/ml GH treatment on P-STAT5 (n = 3); ** P < 0.01, * P < 0.05 vs. untreated cells (untr); Kruskal-Wallis with Dunn's test for multiple comparisons.*

Fig. 4.2

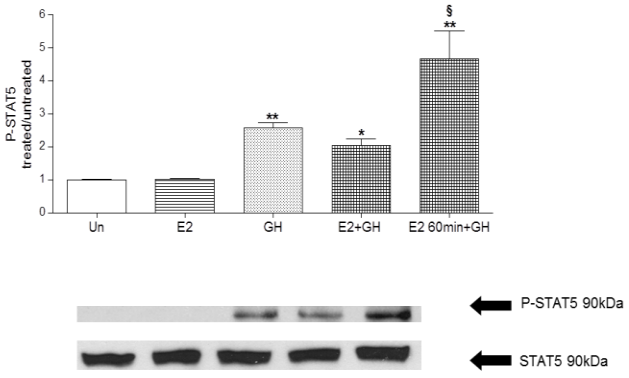


Fig.4.2. *The effect of 60 min GH (5ng/ml) or E2 (10-8M) treatment on STAT5 phosphorylation (P-STAT5). For the combined treatment with the two hormones, E2 was added at the same time as GH (black bar) or 60 min before (squared bar). No changes in total amount of STAT5 were detected. Representative Western blots and relevant quantification, as described in Materials and Methods, (n = 9); ** P < 0.01 vs. untreated cells (untr), § P < 0.05 vs. GH; Kruskal-Wallis with Dunn's test for multiple comparisons.*

4.2 Effect of E2 on GH-related gene expression

As P-STAT5 activates the transcription of GH-related genes, the mRNA expression of OPN, BSP, and IGF2 was measured 6 h after GH (5 ng/ml) or E2 (10^{-8} M). GH induced significant increases in OPN ($P< 0.01$), BSP ($P< 0.05$), and IGF2 ($P< 0.05$) mRNAs. E2 (10^{-8} M) pre-treatment 60 min before GH further increased the effect of GH on OPN, BSP, and IGF2 expression (Fig. 4.3), indicating that E2 exerts a positive modulatory action on GH-induced gene expression in hOBs.

Fig. 4.3

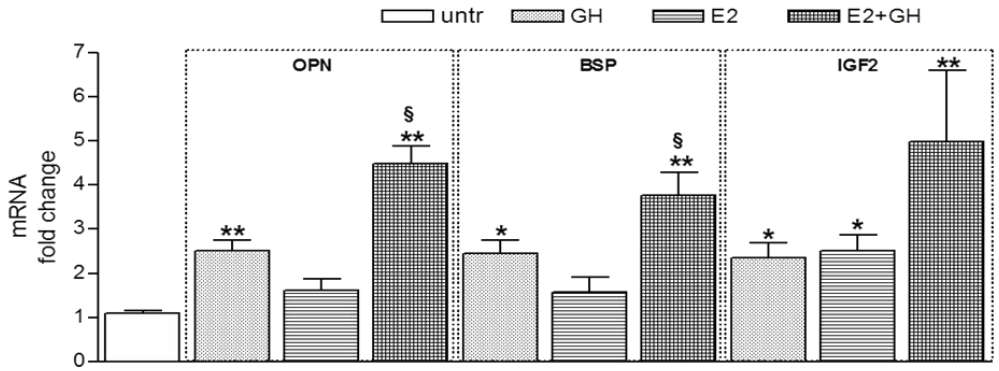


Fig. 4.3. *Osteopontin (OPN), bone sialoprotein (BSP), and IGF2 mRNA expression measured 6 h after GH (5 ng/ml) or E2 (10^{-8}M) treatment in hOBs. In the combined treatment with the two hormones, hOBs were pre-treated with E2 60 min before GH (n = 6). ** P < 0.01, * P < 0.05 vs. untreated cells (untr), § P < 0.05 vs. E2 and vs. GH; Kruskal-Wallis with Dunn's test for multiple comparisons.*

4.3 Effects of E2 on SOCS proteins at 1 h

Considering the role of SOCS proteins as feedback inhibitors of GH signalling, I evaluated the effects of E2 on GH pathway inhibitors, SOCS1, SOCS2, and SOCS3. I analysed both mRNAs and protein levels. Treatment for 60 min with E2 (10^{-8} M) did not modify neither protein levels nor mRNA of SOCS1 and SOCS3 in hOBs (Fig.4.4 A&B), while E2 significantly reduced SOCS2 protein level (Fig. 4.4A) without affecting its mRNA level (Fig.4.4B).

Fig. 4.4

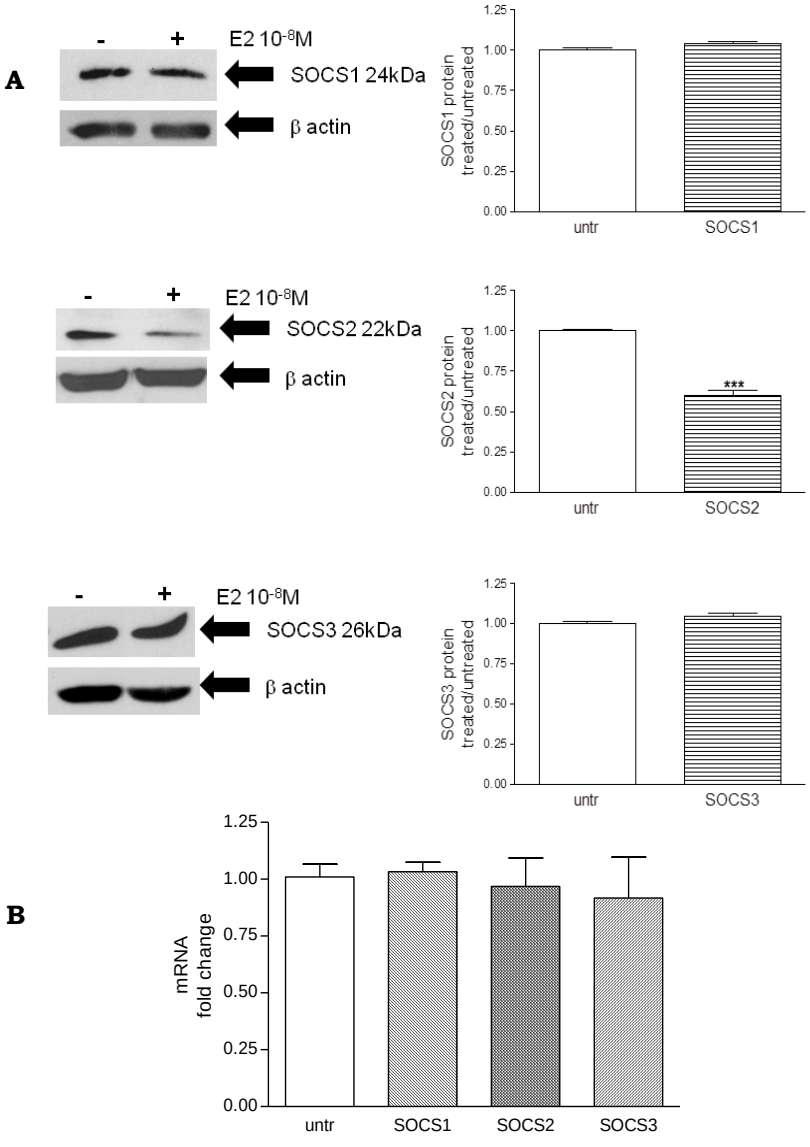


Fig. 4.4A: *SOCS1, 2, 3 protein levels measured after 60 min E2 (10^{-8}M) treatment (representative Western blots and relevant quantifications; n = 3).*

B: *SOCS1, 2, 3 mRNA expression after 60 min E2 (10^{-8}M) treatment in hOBs (n = 6). *** P < 0.001, ** P<0.01 vs. untreated cells (untr); Kruskal-Wallis with Dunn's test for multiple comparisons.*

E2-induced decrease in SOCS2 protein was concentration dependent, being statistically significant from 10^{-10}M (P<0.05) to 10^{-7}M (P<0.001) (Fig. 4.5A) and time-dependent, becoming significant after 30 min (P<0.05) of treatment and lasting until 90 min (P<0.001) (Fig. 4.5 B).

Fig. 4.5

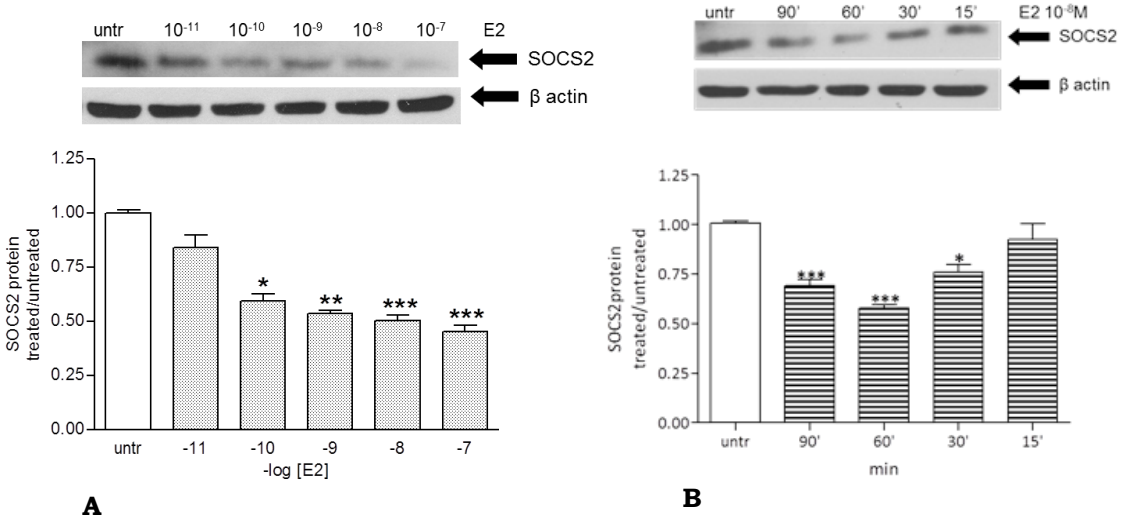


Fig. 4.5 A: *Dose-dependent decrease of SOCS2 protein levels after 60 min treatment with different concentrations of E2 (10^{-11}M-10^{-7}M) (representative Western blots and relevant quantification, as described in the Materials and Methods; n = 4).* ***B:*** *Time-dependent effect of 10^{-8}M E2 treatment on SOCS2 protein levels (n=3). *** P < 0.001, ** P<0.01, * P<0.05 vs. untreated cells (untr); Kruskal-Wallis with Dunn's test for multiple comparisons*

In human skin fibroblasts E2 10^{-8}M was not able to induce any effect on SOCS2 protein levels (Fig. 4.6), suggesting that E2-induced SOCS2 protein decrease is cell-specific.

Fig. 4.6

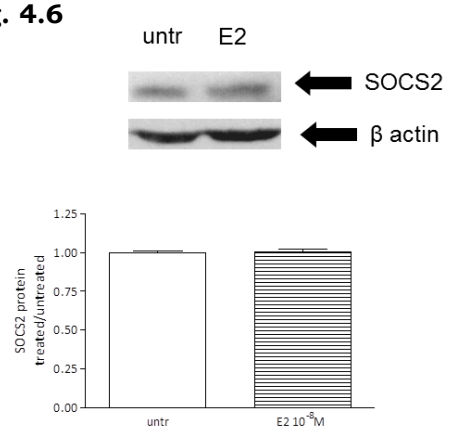


Fig. 4.6:*The effect of 60 min 10^{-8}M E2 treatment on SOCS2 protein levels in skin derived fibroblasts (representative Western blots and relevant quantification, as described in the Materials and Methods; n = 3). E2 does not modify SOCS2 protein levels in these cells.*

In hOBs neither 60 min treatment with GH (5ng/ml) or E2 (10^{-8}M) nor the combined treatment with the two hormones induced any modification of SOCS2 mRNA (Fig. 4.7A), whereas E2 pre-treatment 60 min before GH was able to induce a significant reduction of SOCS2 protein levels (fig. 4.7B).

Fig. 4.7

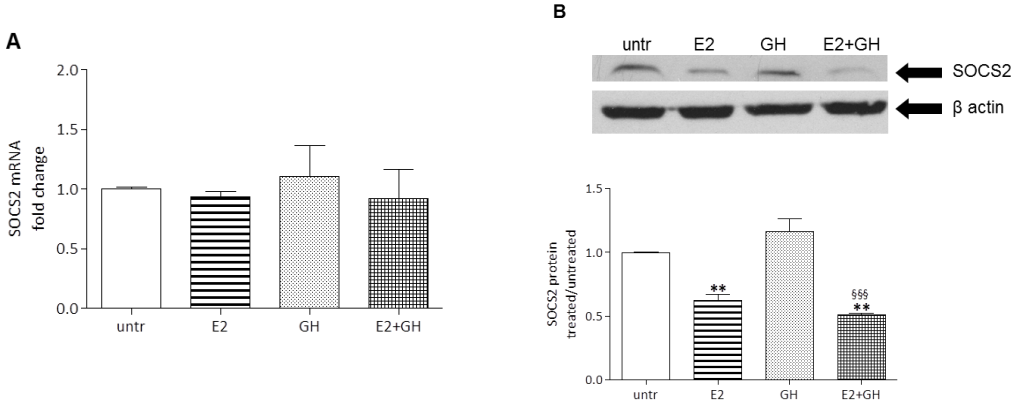


Fig. 4.7 *: The effect of 60 min GH (5ng/ml)*

*or E2 (10^{-8}M) treatment on SOCS2 mRNA expression (A, n=4) or protein levels (B, n=3) in hOBs measured by Western blot and relevant quantification. For the combined treatment with the two hormones, E2 was added 60 min before GH. ** P<0.01, *p<0.05 vs untreated cells (untr); §§§ P<0.001 vs GH; Kruskal-Wallis with Dunn's test for multiple comparisons.*

In order to evaluate the involvement of SOCS2 in E2 positive modulation of GH signalling, hOBs were transfected with small interference RNA (siRNA) targeting SOCS2. The reduction in SOCS2 mRNA and protein level obtained in transfected hOBs is shown in Fig. 4.8A and B, respectively.

Fig. 4.8

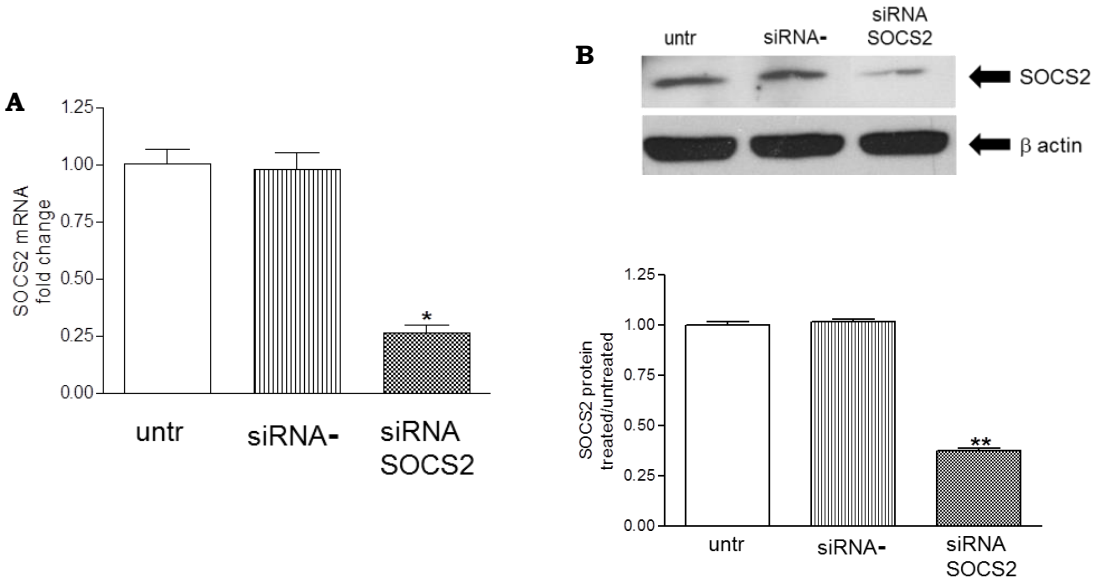


Fig. 4.8. *The effect of the transfection with the siRNA targeting SOCS2 gene expression or scrambled control (siRNA-) of primary hOBs on* ***A:*** *SOCS2 mRNA expression assessed by real time PCR to confirm gene knockdown (n=3) and* ***B:*** *SOCS2 protein levels measured by Western blot and relevant quantification (n=3). **P<0.01, * P<0.05 vs. siRNA-; Mann-Whitney test.*

In cells lacking the negative feedback of SOCS2 on GH signalling, GH induced a greater amount of P-STAT5. Sixty min E2 pre-treatment of hOBs silenced for SOCS2 was not able to induce any potentiating effect on GH induced STAT5 phosphorylation (Fig.9A). In hOBs treated with the non-coding siRNA negative control, E2 potentiating effect on GH induced P-STAT5 was preserved (Fig. 9A). In SOCS2 silenced hOBs, the positive effect of E2 pre-treatment on BSP and OPN mRNA expression induced by GH was no longer present (Fig.4.9B).

Fig. 4.9

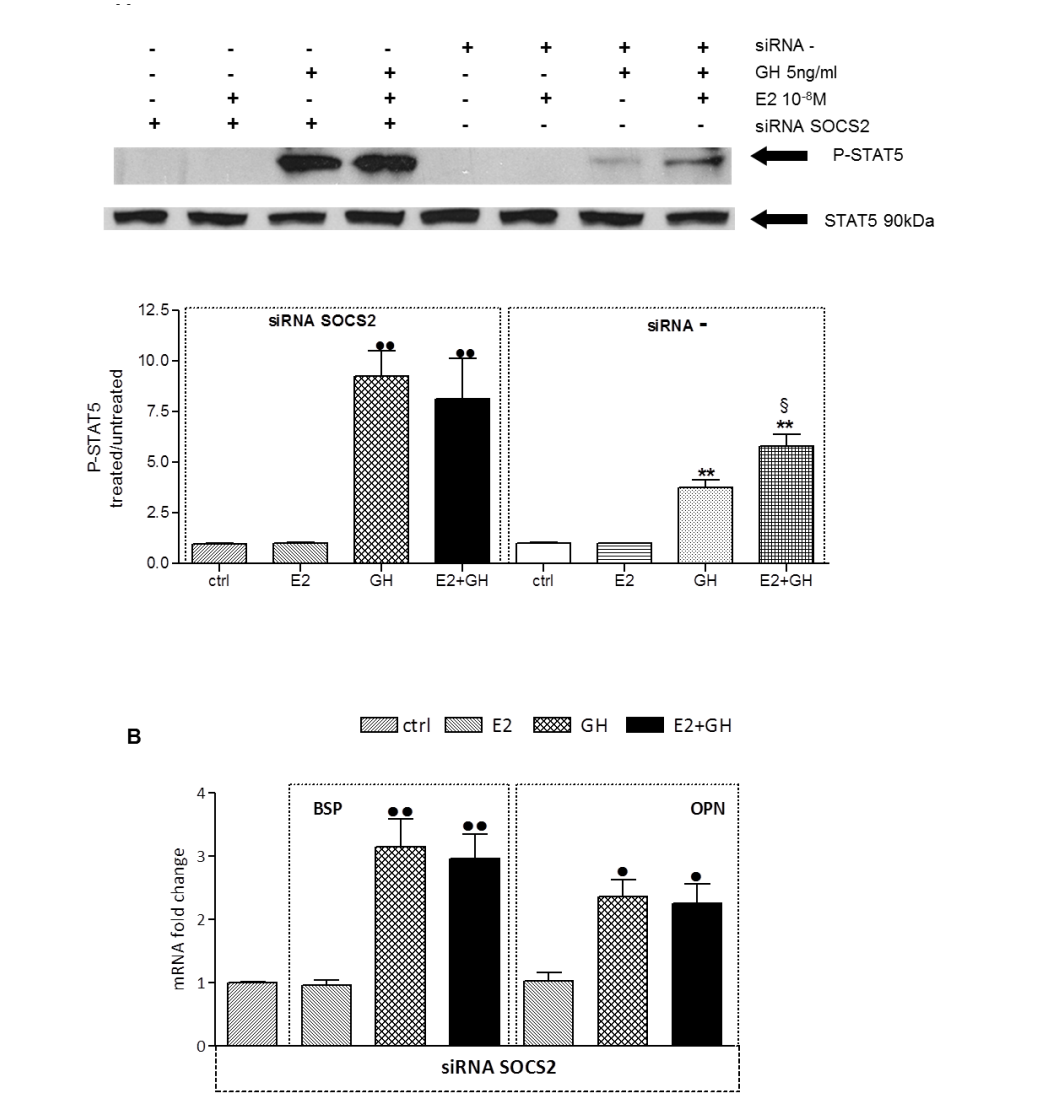


Fig. 4.9 A: *The effect of 60 min E2 (10^{-8}M) pre-treatment on GH (5ng/ml) induced STAT5 phosphorylation (P-STAT5) in hOBs transfected with siRNA targeting SOCS2 gene expression (ctrl = siRNA SOCS2, hatched bars, n= 4) or with siRNA non coding control (siRNA-) (ctrl = non coding siRNA; white bar, n= 4);* ***B:*** *60 min E2 pre-treatment effect on GH induced BSP and OPN mRNA expression in silenced cells (n= 4). **P<0.01, * P<0.05 vs siRNA- (ctrl), § P<0.05 vs. GH; •• P<0.001 vs ctrl; Kruskal-Wallis with Dunn's test for multiple comparisons*

In order to assess whether E2 potentiating effect on GH signalling requires *de novo* gene transcription, hOBs were pre-incubated for 30 min with actinomycin D before E2 treatment for 60 min. E2 was able to reduce significantly SOCS2 protein levels also in presence of the inhibitor of transcription, suggesting that this E2 effect is non-genomic (Fig. 4.10).

Fig. 4.10

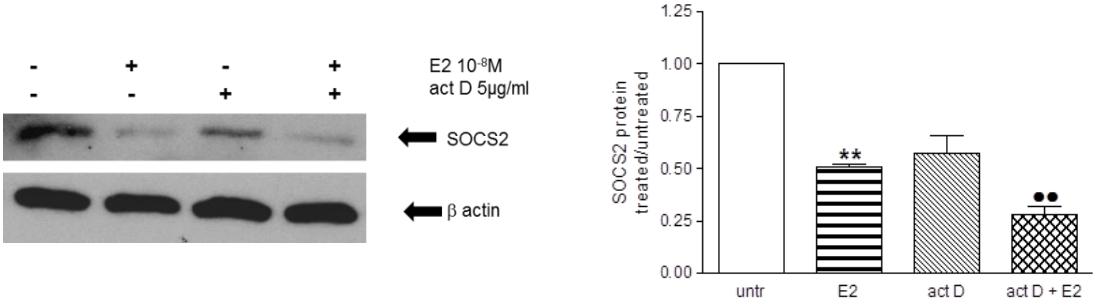


Fig. 4.10: *The effect of 30 min actinomycin D (act D, 5µg/ml) pre-treatment before E2 treatment for 60 min on SOCS2 protein levels in hOBs (representative Western blot and relevant quantification; n = 6). ** P<0.01 vs untreated cells (untr), •• P <0.01 vs actinomycin D; Mann-Whitney test.*

4.4 Effect of E2 on SOCS2 degradation

On the basis of these results I investigated whether E2 was able to facilitate SOCS2 degradation by evaluating the effect of E2 on SOCS2 ubiquitination. Sixty min E2 (10^{-8} M) treatment was able to significantly increase the amount of ubiquitinated SOCS2 protein (Fig. 4.11A). In parallel, non-ubiquitinated SOCS2 protein amount measured in the cell lysates after the immunoprecipitation of the ubiquitinated proteins was significantly reduced by E2 treatment (Fig.4.11B). Pre-treatment with the proteasome inhibitor MG132 (5 μM) 20 min before E2 prevented the E2-induced reduction in SOCS2 protein (Fig. 4.12). These results suggest that E2 amplifies intracellular GH signalling by favouring SOCS2 degradation through the proteasome machinery.

Fig. 4.11

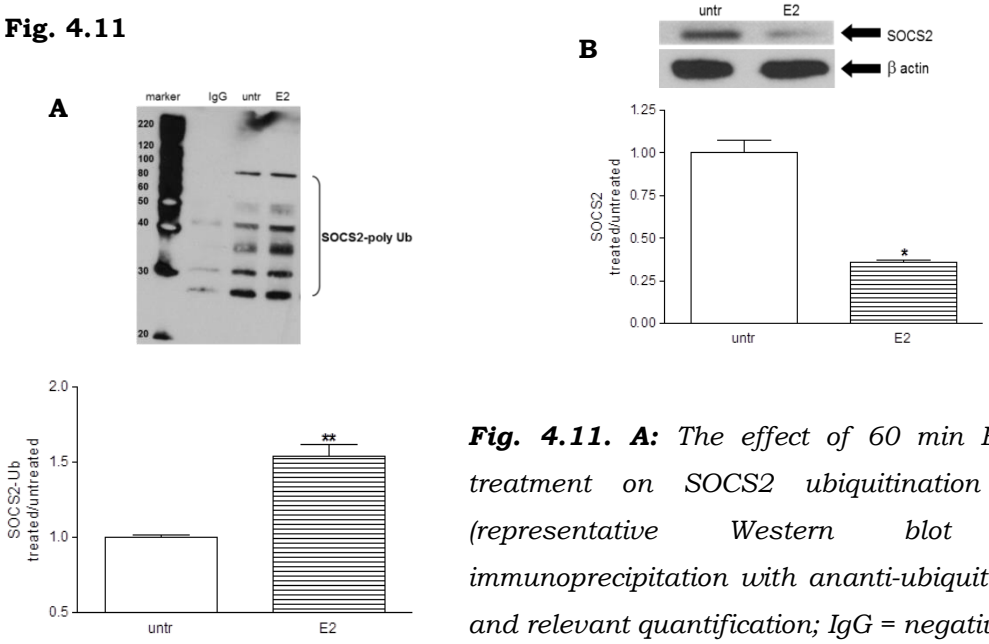


Fig. 4.11. A: *The effect of 60 min E2 (10^{-8} M) treatment on SOCS2 ubiquitination in hOBs (representative Western blot following immunoprecipitation with ananti-ubiquitin antibody and relevant quantification; IgG = negative control; n=6). Multiple bands indicate poly-ubiquitination of SOCS2. E2 treatment induced an increase in the total ubiquitinated SOCS2 levels.* ***B:*** *Non ubiquitinated SOCS2 protein measured in the cell lysate after immunoprecipitation of the ubiquitinated proteins as indicated above are decreased after E2 treatment (representative Western blot and relevant quantification, n = 6) . ** P < 0.01, * P < 0.05 vs. untreated cells (untr); Mann-Whitney test.*

Fig. 4.12

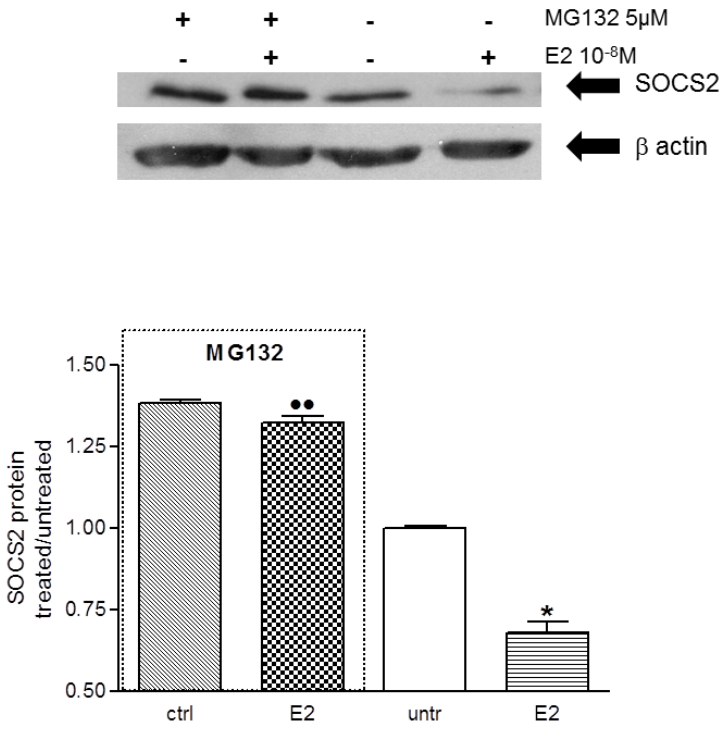


Fig. 4.12. *The effect of 20 min pre-treatment with the proteasome inhibitor MG132 (5 μM) in hOBs on the reduction of SOCS2 protein induced by 60 min E2 (10^{-8}M) treatment (n=7). MG132 prevents the inhibitory action of E2 on SOCS2 protein levels. (Representative Western blot and relevant quantification). * P < 0.05 vs. untreated cells (untr); •• P < 0.01 vs. E2 alone; Kruskal-Wallis with Dunn's test for multiple comparisons.*

4.5 Long term effect of E2 treatment on STAT5 phosphorylation and SOCS2 levels

Having identified a positive role of 60 min E2 pre-treatment on GH pathway, I decided to evaluate if the observed effect is maintained over time. For this purpose we treated hOBs for 3h with E2 before giving GH. After 3h of E2 pre-treatment there was still a significant enhancing effect on STAT5 phosphorylation measured after 60 min of GH treatment ($P < 0.05$) compared to GH alone (Fig. 4.13)

Fig. 4.13

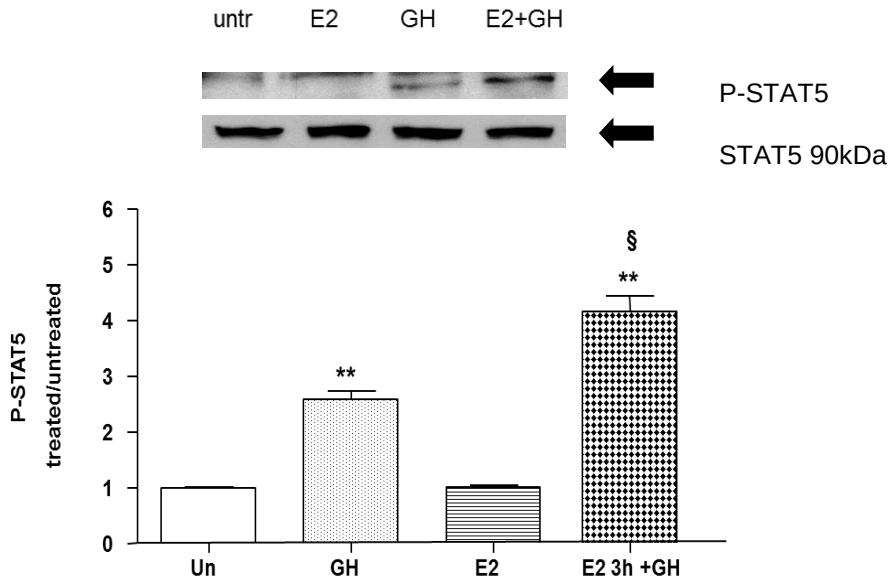


Fig. 4.13 *The effect of 60 min GH (5ng/ml) or E2 (10^{-8}M) treatments on STAT5 phosphorylation (P-STAT5) in hOBs. For the combined treatment with the two hormones (squared bar), E2 was added 3h before. No changes in total amount of STAT5 were detected. Representative Western blots and relevant quantification, as described in Materials and Methods, (n =3); ** P < 0.01 vs. untreated cells (untr), § P < 0.05 vs. GH; Kruskal-Wallis with Dunn's test for multiple comparisons.*

After 3h E2 (10^{-8}M) pre-treatment, there was a reduction in SOCS2 protein levels (Fig 4.14 A), but differently to 60 min E2 pre-treatment, this time there was a corresponding decrease in SOCS2 gene expression (Fig. 4.14 B).

Fig. 4.14

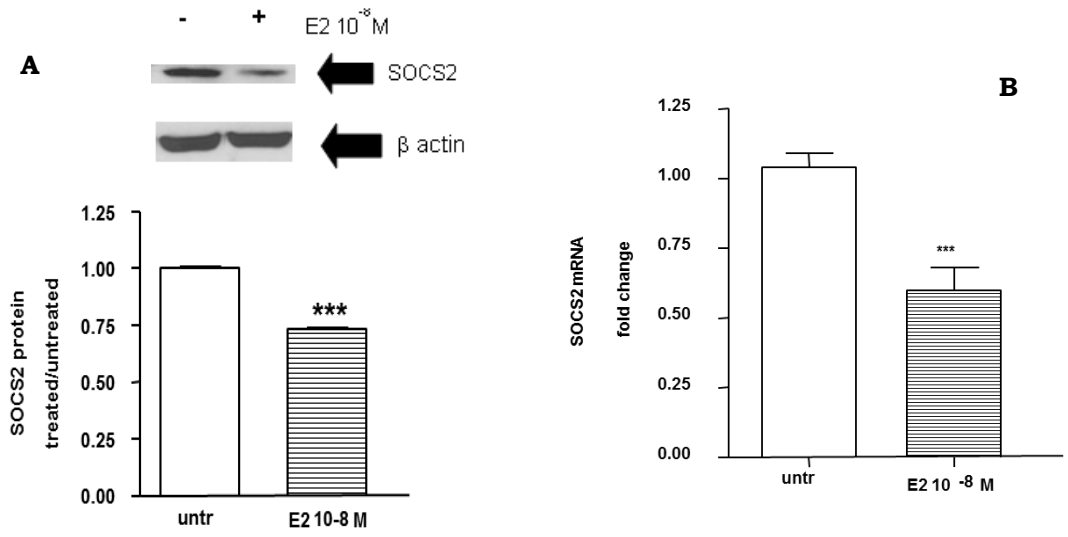


Fig. 4.14A: *SOCS2 protein levels measured after 3h E2 (10^{-8} M) treatment (representative Western blots and relevant quantifications; n = 3).* ***B:*** *SOCS2 mRNA expression after 60 min E2 (10^{-8} M) treatment in hOBs (n = 3). *** P < 0.001, ** P<0.01 vs. untreated cells (untr); Kruskal-Wallis with Dunn's test for multiple comparisons.*

Moreover cell treated for 3h with E2 in the presence of actinomycin D showed no differences in SOCS2 protein levels compared to the relevant control, thus suggesting an involvement of a genomic mechanism (Fig. 4.15).

Fig. 4.15

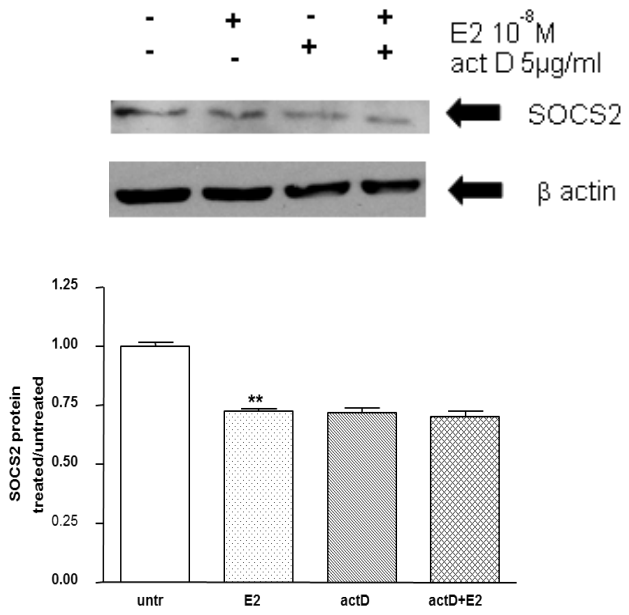


Fig. 4.15: *The effect of 30 min actinomycin D (act D, 5µg/ml) pre-treatment before E2 treatment for 3h on SOCS2 protein levels in hOBs (representative Western blot and relevant quantification; n = 6). ** P<0.01 vs untreated cells (untr,; Mann-Whitney test.*

4.6 Effect of Raloxifene and Tamoxifen pre-treatments on GH-induced STAT5-phosphorylation and on SOCS2 levels

At this point of the research, considering that Selective Estrogen Receptor Modulator (SERMS), are endowed of estrogen agonist activity in bone and antagonist activity in other tissues, I evaluated if they behave like E2 on GH pathway in hOBs.

Cells were stimulated 60 min with Raloxifene (Ral, 10^{-8}M) or Tamoxifen (Tam, 10^{-10}M) before giving GH and the level of PSTAT5 was evaluated. Sixty min pre-treatment with Ral or Tam did not induce a significant increase of P-STAT5 compared with GH alone (Fig 4.16 A& B); Both SERMS *per se* had no effect on P-STAT5 levels. Total STAT5 levels were not affected by Ral or Tam treatment (Fig. 4.16C). I then investigated the effect of both Ral and Tam on SOCS2 gene transcription after 3h. None of the two treatments inhibited SOCS2 gene expression (Fig 4.17), suggesting that these two SERMs do not share these E2 activities in bone cells.

Fig. 4.16 A

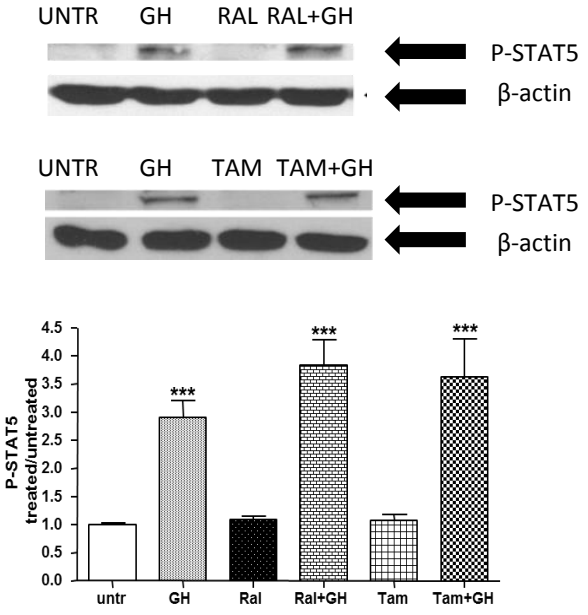
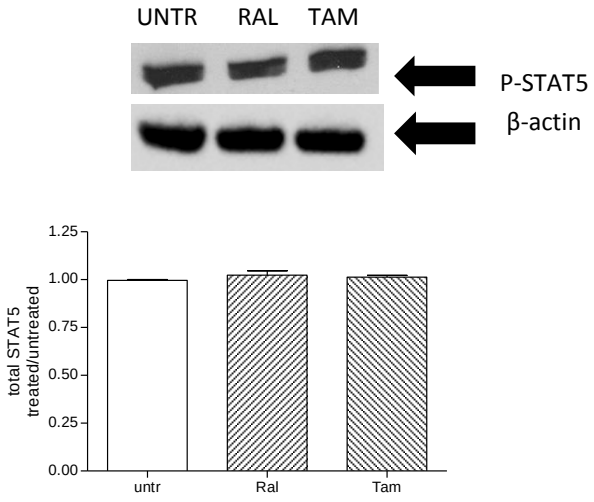


Fig. 4.16 A *The effect of 60 min GH (5ng/ml), Ral (10^{-8}M), or Tam (10^{-10}M) treatment on STAT5 phosphorylation (P-STAT5). For the combined treatment with Ral/Tam and GH, SERMS were added 60 min before. Representative Western blots and relevant quantification,as described in Materials and Methods, (n =3); *** P < 0.01 vs. untreated cells (untr), Kruskal-Wallis with Dunn's test for multiple comparisons.*

Fig. 4.16 B



B *No changes in the total amount of STAT5 were detected; representative Western blots and relevant quantification, as described in Materials and Methods, (n =3)*

Fig. 4.17

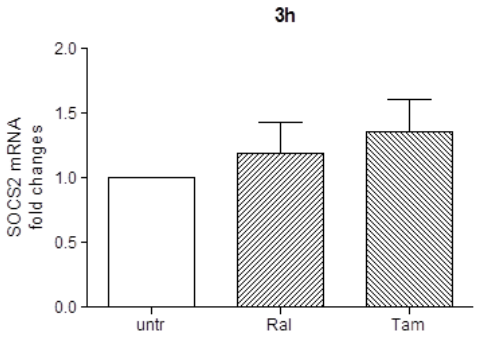


Fig 4.17 *SOCS 2 mRNA expression after 3h Ral (10^{-8} M) or Tam (10^{-10}M) treatment in hOBs (n = 6). Effect not significant vs. untreated cells (untr); Kruskal-Wallis with Dunn's test for multiple comparisons.*

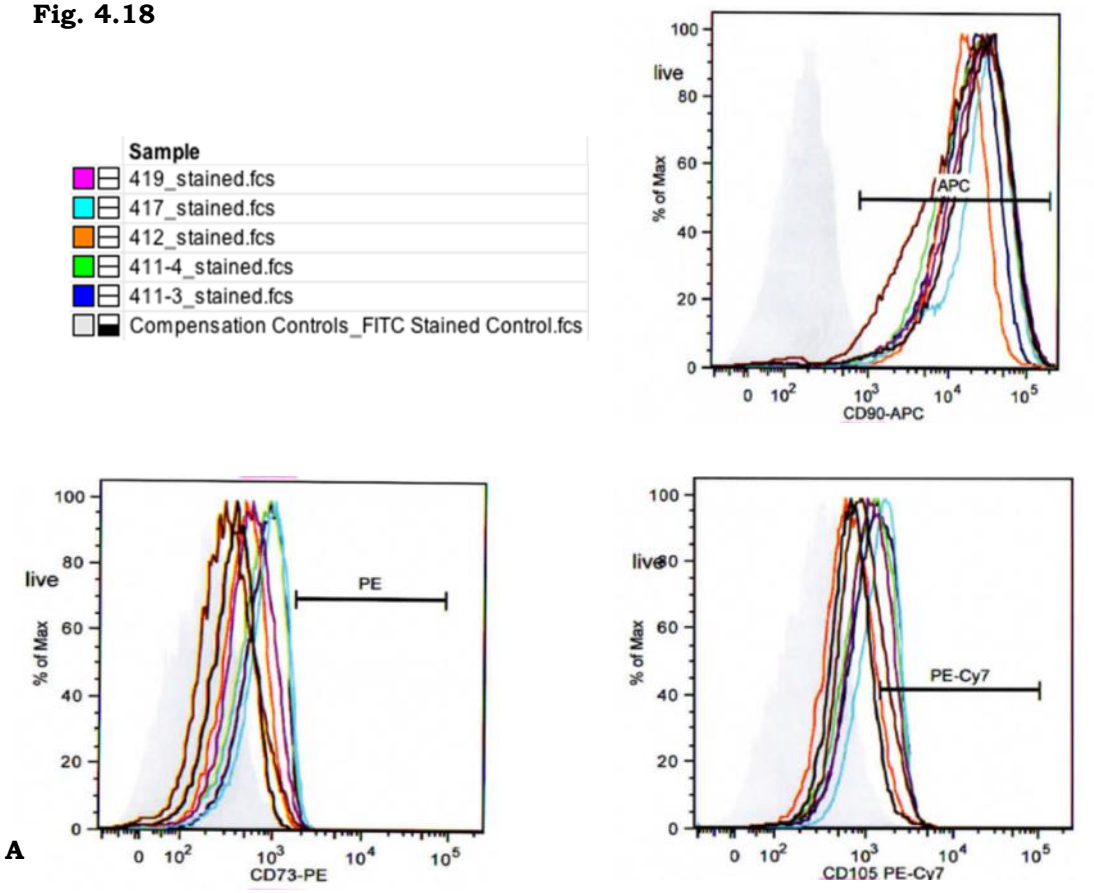
4.7 Characterization of human stromal mesenchymal stem cells (hMSCs) derived from trabecular bone specimens and evaluation of their differentiation capability

Besides mature osteoblasts also their mesenchymal precursors are important for the maintenance of bone mass. Therefore I decided to evaluate GH and E2 interaction in human mesenchymal stem cells. For this purpose hMSCs were isolated from trabecular bone according to Zhou et al, 2008 protocol (see Material and Methods). I performed a FACS analysis in order to assess the presence of CD90, CD73 and CD105, and the lack of CD34 and CD45.

An average of 75.5% of the cells isolated stained positive for CD90 and around 64% were simultaneously positive for CD73 and CD105. Overall 46% of the alive population stained positive for all the three CDs. In this triple positive population I did not find CD34+ and CD45+ cells (average less than 0.1%), which identify hematopoietic precursor cells (Fig. 4.18 A&B).

After isolation and FACS analysis, cells were tested for their differentiation capability by cultivating them in osteogenic or adipogenic medium for 14 days. In both the conditions cells were able to differentiate into the osteogenic or adipogenic lineage as shown respectively by Alizarin Red and Oil red O stainings, thus confirming the pluripotency potential of these cells (Fig 4.19 A& B).

Fig. 4.18



A

B

Sample	% CD90+	→	% CD73+CD105+	% + tot live	→	CD34+CD45+
411_3	97.5		51.6	50.4		0,14
411_4	96.5		43.4	41.9		0,2
412	65.4		80.6	52.7		0,01
417	56		57.4	32.2		0
419	62		85.1	52.7		0

Fig.4.18 ***A*** *Representative FACS analysis of 4 different samples of hMSCs stained For CD90, CD105 and CD73 compared with a stained compensation control.*

B *percentages of the total living cells triple positive.*

Fig. 4.19

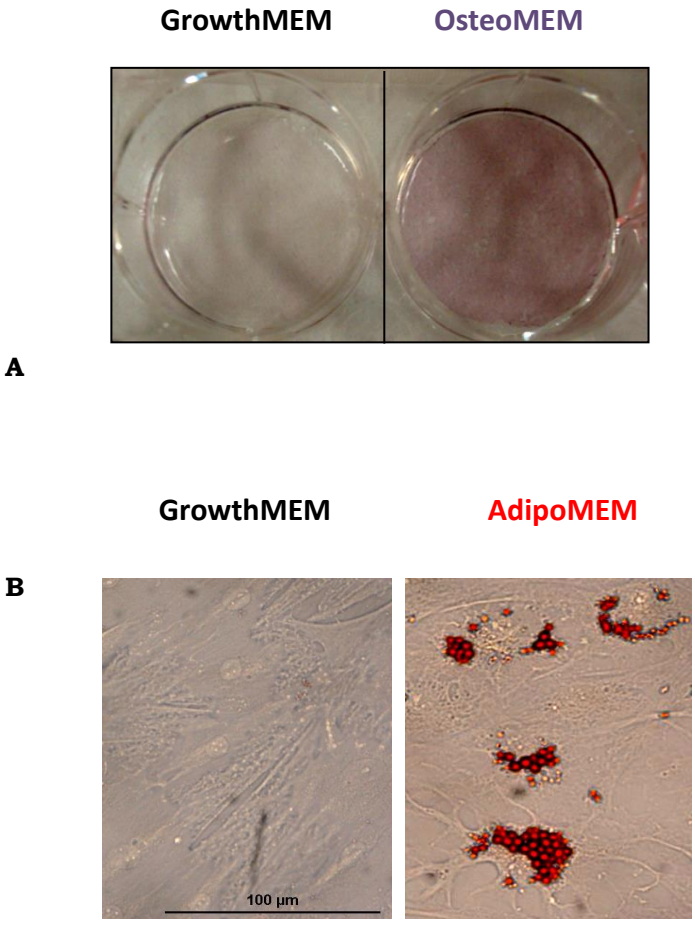


Fig. 4.19 A *Alizarin red staining ofhMSCs cultured 14 days in osteogenic medium (right panel) or in normal growth medium (left panel), 6 well plate* ***B*** *Oil Red O staining of hMSCs cultured 14 days in adipogenic medium (right panel) or in normal growth medium (left panel), 20x magnification*

4.8 Effects of E2 on GH intracellular signaling in hMSC

I then analysed E2-GH post-receptor interaction in hMSCs by applying the same experimental schedules of the hOBs experiments. Sixty min E2 (10^{-8}M) pre-treatment induced a significant increase (P<0.01) of P-STAT5 compared with GH alone whereas E2 (10^{-8}M) *per se* had no effect on P-STAT5 levels (Fig. 4.20). Moreover 60 min E2 treatment decreased the protein levels of SOCS2, (Fig. 4.21) indicating that E2 enhances GH signalling at post-receptor level, as it was observed in hOBs.

Fig. 4.20

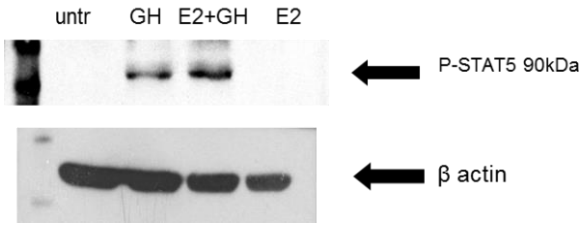


Fig. 4.20: *The effect of 60 min GH (5ng/ml) or E2 (10^{-8}M) treatment on STAT5 phosphorylation (P-STAT5) in hMSCs. For the combined treatment with the two hormones, E2 was added 60 min before GH.*

*No changes in the total amount of STAT5 were detected (data not shown). Representative Western blots and relevant quantification. ** P < 0.01 vs. untreated cells (untr), • P < 0.05 vs. GH; Kruskal-Wallis with Dunn's test for multiple comparisons.*

Fig. 4.21

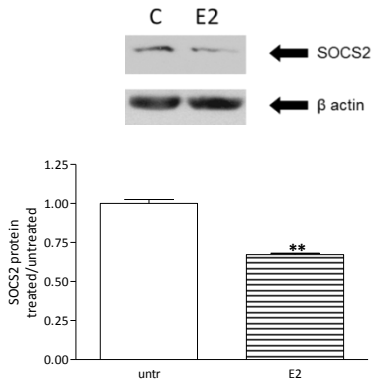


Fig. 4.21:*The effect of 60 min 10^{-8}M E2 treatment on SOCS2 protein levels in hMSCs, C= untreated cells. Representative Western blot and relevant quantification (n=3);** P<0.01vs untr; Mann-Whitney test*

4.9 Validation of the hMSC model after GH long term treatment

I used hMSCs isolated from trabecular bone to verify the effect of the long term treatment of GH (5ng/ml; 3 times/week for14 days) on gene expression. GH was able to shift hMSCs towards osteogenesis, as shown by the real-time PCR results. GH significantly increased the transcription of OPG and OSX, confirming a pro-osteogenic effect of GH on hMSCs. There was a trend to increase RUNX2 that did not reach statistical significance whereas ADN and C/EBPα were not affected (Fig. 4.22 A&B).

Fig. 4.22

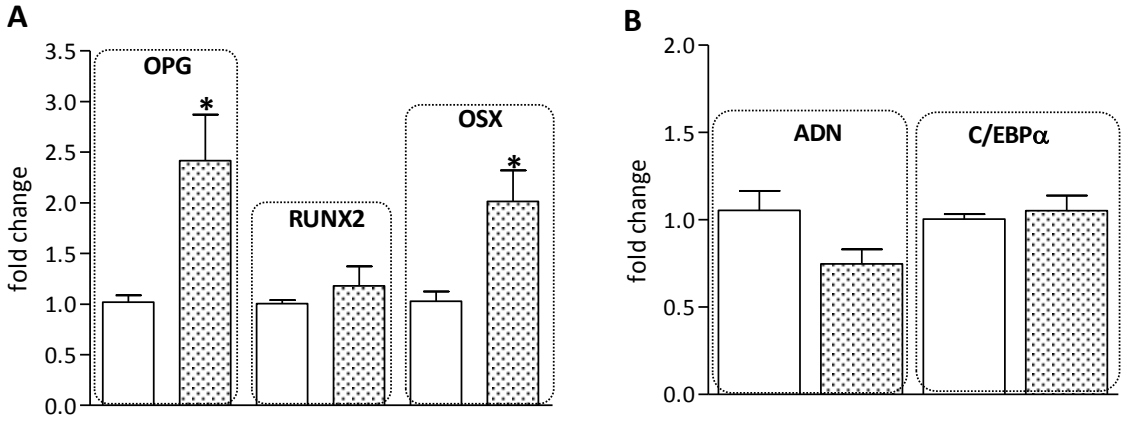


Fig. 4.22.A*Osteoprotegerin (OPG) and Osterix (OSX) mRNA expression measured 14 days after chronic GH treatment of hMSCs (5 ng/ml,3 times/week) cultivated in growth medium. * P < 0.05 vs. untreated cells (C); Kruskal-Wallis with Dunn's test for multiple comparisons.* ***B*** *Adiponectin (ADN) and C/EBPa mRNA expression measured in the same conditions.*

To further characterize GH role in hMSC differentiation, I treated the cells with GH cultivated in a medium favouring adipogenesis (adipoMEM) for 14 days. GH was able to drastically reduce adipocytes formation as shown by Oil Red O

staining (Fig. 4.23 A). This observation was confirmed by the relative quantification of the staining (Fig 4.23B). Concomitantly GH treatment decreased significantly the expression of adiponectin (ADN) and C/EBPα (Fig. 4.23 C) confirming antiadipogenic effect of GH in these types of cells.

Fig. 4.23

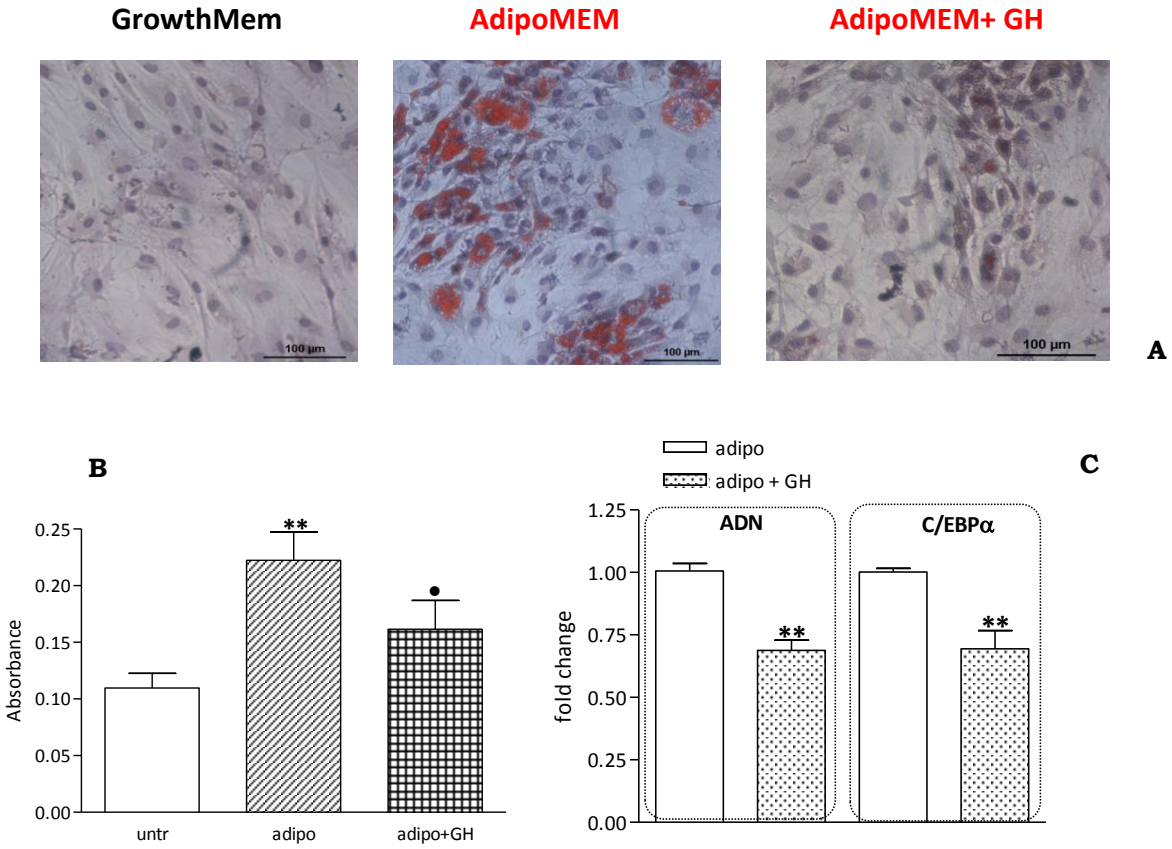


Fig.23 A *Oil Red O staining of hMSCs cultured 14 days in normal growth medium (left panel), adipogenic medium (central panel), adipogenic medium with GH (5ng/ml, 3 times/week, right panel), 10x magnification.* ***B****Oil Red O staining quantification. Data are expressed as a ratio to the DNA amount of the relevant sample. ** P < 0.01 vs. untreated cells (untr), • P < 0.05 vs. adipoMEM; Kruskal-Wallis with Dunn's test for multiple comparisons.* ***C*** *Adiponectin (ADN) and C/EBPa mRNA expression measured 14 days after chronic GH treatment of hMSCs (5 ng/m, 3 times/week) in adipoMEM; ** P < 0.01 vs adipoMEM cells; Mann-Whitney test.*

4.10 Effects of long term GH treatment on miRNA expression in hMSCs

Considering the relevance of microRNAs in MSC differentiation, we evaluated the possible changes in the expression of miR-29c, mirR-22, which are known to favour osteogenesis and miR-204 which promotes adipogenesis, after GH chronic treatment. For this purpose, hMSC cultured in normal growthMEM were treated 3times/week with GH (5ng/ml) for 14 days. Accordingly to its pro-osteogenic activity, GH increased the expressions of miR-22 ($P<0.01$) and miR-29c ($P<0.05$) in hMSC cultured in growthMEM and it had no effect on miR-204 (Fig. 4.24).

Fig. 4.24

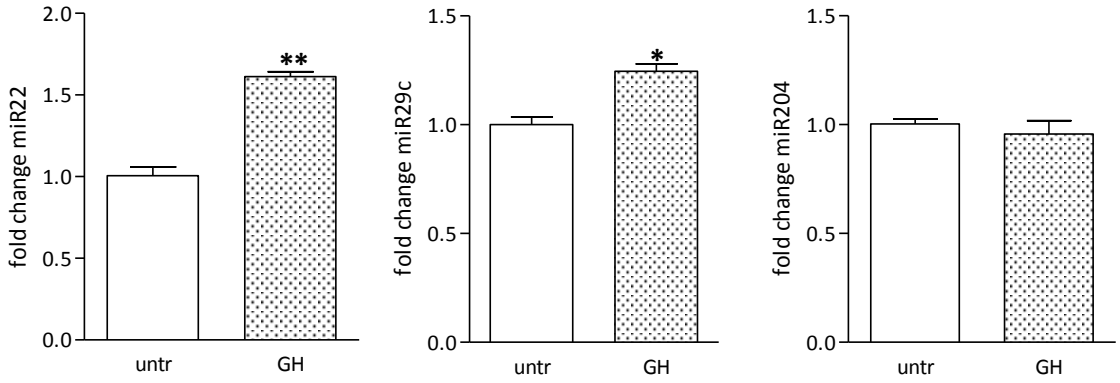
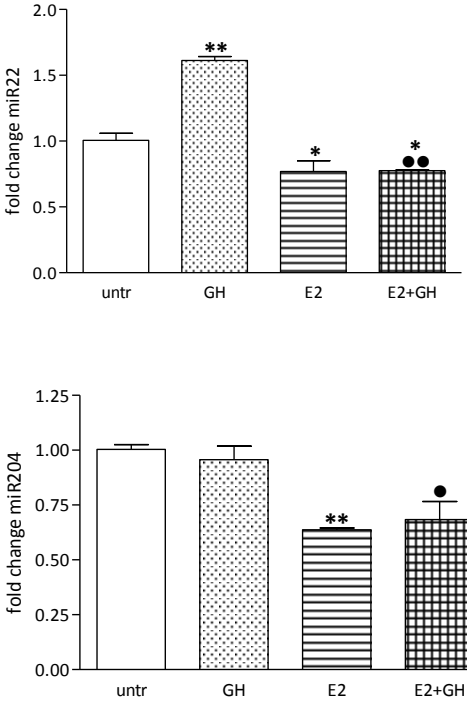


Fig. 24: *miR-22 (left panel), miR-29c (central panel) and miR-204(right panel) expression measured 14 days after chronic GH treatment of hMSCs (5ng/ml,3 times/week) in normal growth medium.* P < 0.05; **P<0,01 vs untreated (untr) cells. Mann-Whitney test.*

4.11 Effects of the combined treatment of E2 and GH on microRNAs in hMSCs.

Next I investigated the possible interaction E2-GH on the expression of the previously evaluated miRNAs in hMSC. Cells were treated with E2 (10^{-8}M) or GH (5 ng/ml) for 14 days 3 times/week. In the combined treatment with the two hormones, E2 was always given 60 min before GH. E2 treatment *per se* inhibited the expression of all the microRNAs analyzed, miR-22, miR-29c and miR-204. E2 pre-treatment was able to inhibit the stimulatory effect of GH on miR-22 and miR-29c. E2 decreased miR-204 was maintained also in the presence of GH (Fig. 4.25). These results suggest that E2 negative effect on miRNA expression *per se* covers the positive modulation of GH-induced STAT5 phosphorylation.

Fig. 4.25



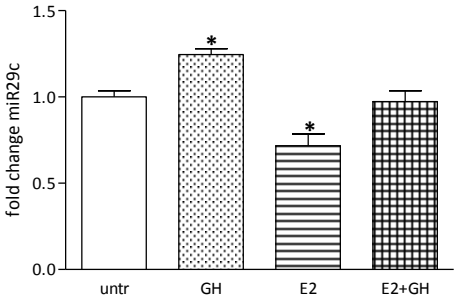


Fig. 25: *miR-22 (upper left panel) miR-29c (upper right panel) and miR-204 (lower panel) expressions measured 14 days after GH (5ng/ml), E2 (10^{-8}M), E2+GH treatments (3 times/week) in normal growth medium. In the combined treatment E2 was given 60 min before GH;* P < 0.05, ** P <0.01 vs untreated cells; •P<0.05; •• P < 0.01 vs. GH.*

5. Discussion

The present study showed for the first time a positive modulation of GH intracellular pathway, evaluated as increase in STAT5 phosphorylation, by E2 pre-treatment, both in hOBs and hMSCs.

5.1 GROWTH HORMONE AND 17β-ESTRADIOL IN hOBs

In human osteoblasts GH activated the JAK2/STAT5 cascade as in other cells types (Leung et al., 2003, Leong et al., 2004). GH concentrations, within the physiological range, elicited a significant and dose-dependent phosphorylation of STAT5, which in turn activated the transcription of the GH-responsive genes IGF2, OPN and BSP.

In vitro human osteoblasts, in response to GH, do not produce IGF1, but IGF2 (Mrak et al 2007). Although IGF1 and IGF2 share the same anabolic features, (Froesch et al., 1976), there is increasing evidence that IGF2 plays a critical role in the maintenance of bone homeostasis favouring both osteogenic differentiation (Hamidouche Z, 2010) and osteoclastogenesis (Fukuoka H, 2005; Nakao K, 2009). IGF2 production by osteoblasts could modulate the bone microenvironment through an autocrine/paracrine mechanism in order to create the right conditions for the osteoblasts-osteoclasts coupling and therefore for a balanced bone turnover. OPN and BSP are proteins secreted by mature osteoblasts involved in matrix deposition and mineralization. Their role is not related exclusively to the regulation of mineral turnover; they cooperate also to the modulation of bone homeostasis. Global null mice for OPN show indeed an altered osteoclastogenesis (Franzén et al., 2008) and an impairment in MSCs osteogenic/adipogenic commitment (Chen et al, 2013); mice KO for BSP display impaired osteoblast and osteoclast function as well (Wade-Gueye NM, 2010). Nevertheless, the molecular mechanisms by which OPN and BSP control cells metabolism and differentiation have not been yet elucidated. E2 through the enhancement of the GH induced production of

IGF2, OPN, BSP could further promote the maintenance of the physiological homeostasis of bone.

A previous work of Leung and colleagues (2003) demonstrated that E2 reduces GH induced STAT5 phosphorylation in hepatoma and breast cancer cells. The opposite effects observed by Leung study and my data could be ascribed to the different cell types used: primary human bone cells in this study, instead of transfected tumor cell lines as used by Leung's group. The different cells used may also explain the different GH concentrations used for inducing STAT5 phosphorylation: GH 500ng/ml for cancer cells versus GH 5ng/ml for primaries. Investigating hormonal interaction in non-transformed cells in vitro gives the advantage to work in conditions closer to a physiological situation, thus the concentration used in this study are within the in vivo normal range of GH in humans (Hartman et al., 1991) whereas Leung used a dosage 100 times the physiological hormone levels.

The mechanism by which E2 positively modulates GH is driven by decreasing the amounts of one of its feed-back inhibitor, SOCS2. Three SOCSs are generally involved in inhibiting JAK/STAT signaling), SOCS1, SOCS2 and SOCS3 (Ahmed SF 2010. All three proteins are expressed in hOBs but only SOCS2 in inhibited by E2. The lack of effect on SOCS1 was almost expected considering that SOCS1 was shown to act primarily in the immune system (Starr R, 1998), whereas the lack of effect of E2 on SOCS3 protein is in agreement with previous data obtained in a kidney cell line (Leung KC, 2003). Of the three proteins, SOCS2 is of primary importance in skeletal regulation. Mice KO for SOCS2 display an altered bone phenotype; the mice grow more rapidly, have increased bone growth and longer bone length compared to their wild-type littermates [McRae VE 2009]. They exhibit phenotypic characteristics of both GH and IGF-I transgenic mice [Metcalf D 2000]. Interestingly, SOCS2 overexpression enhances growth. It has therefore been proposed that SOCS2 may exert a dual effect on GH signaling: normal physiological levels of SOCS2 attenuate the pathway, while higher concentrations of SOCS2 might enhance it

by interfering with the activity of other more potent inhibitors (Greenhalgh CJ, 2002; Pass C, 2009).

In hOBs the direct involvement of SOCS2 in E2 induced enhancement of STAT5 phosphorylation was demonstrated by its specific gene silencing. The molecular mechanisms that mediate E2-induced SOCS2 reduction are different depending on the timing of the pretreatment. After 1h E2 stimulus there is no effect on SOCS2 transcription and the reduction in the protein levels is to be ascribed to a non-genomic dependent mechanism. In fact Actinomycin D, that inhibits DNA transcription, did not blunt E2 positive effect on STAT5 phosphorylation. I therefore investigated possible post transcriptional regulation mechanism. I focused on the proteasome degradation of the protein, discovering that E2 stimulates SOCS2 ubiquitination thus favouring its subsequent proteasomal degradation. Indeed pretreatment of cells with the proteasome inhibitor MG132, a cell-permeating molecule that specifically blocks the activity of the 26S proteasome subunit, prevented E2 induced reduction in SOCS2. The ability of E2 to modulate protein stability has been recently shown in different cell models and in different direction. In MCF-7 cells E2 reduces TGF-β signalling by promoting Smad ubiquitination and degradation. This activity of E2 does not require the DNA binding ability of ERα, but occurs through the formation of a protein complex of ERα with Smad and the ubiquitin ligase, Smurf1, which induces the enhancement of Smad ubiquitination and relevant degradation (Ito I, 2010). In other breast cancer cells instead, E2 was shown to modulate the stability of BRCA1, by inhibiting the proteasome degradation through an Akt dependent-phosphorylation of the protein.

After 3h of E2 treatment, the decrease in SOCS2 protein levels I observed, involves a reduction of SOCS2 gene expression. Actinomycin D treatment was able to prevent the E2 effect on protein levels confirming a genomic involvement in E2 effect. Moreover the fact that E2 negative regulation of the GH inhibitor SOCS2 involves an initially rapid protein degradation maintained for a longer time by a decrease in its gene expression strengthens the

importance of this modulation by estrogens for osteoblast activity. It emerges that the estrogen-dependent operative state is a key element of the post-receptor signaling machinery, which is central to the response of bone to GH activity. Moreover there is increasing evidence (Iglesias-Gato et al., 2013) that the responsiveness to GH depends upon circulating sex steroids levels.

Interestingly the previous work of Leung showed that E2 inhibits STAT5 phosphorylation through the up-regulation of SOCS2 expression, mechanism mediated by ER genomic activity, thus suggesting a tissue-specific action of E2 on GH intracellular pathway. Hypothesis confirmed by our data in human primary fibroblasts, where we did not observe any modulation of SOCS2 levels after E2 treatment.

5.2 GROWTH HORMONE AND SERMs IN hOBs

Considering the widespread use in clinics of SERMs I tested the effect of Raloxifene and Tamoxifen on GH signaling. Both SERMs showed a trend to decrease SOCS2 levels after 1h treatment, and to slightly increase GH-induced STAT5 phosphorylation however without reaching the significance. Moreover after 3h treatment of Raloxifene and Tamoxifen there is no modification in the expression of SOCS2. Our data suggest that, despite the estrogen agonist activity of SERMs in bone, Raloxifene and Tamoxifen do not exert the same positive effect on GH signaling as E2. This is not the first time that SERMs in vitro do not act as pure estrogen agonists as expected. Tamoxifen was indeed shown to be both an agonist and antagonist of 17β-estradiol action in osteoblast-like cells (Fournier et al., 1996; Rao et al., 1996) or not to have the same power as E2 in inducing a biological response (Waters et al., 2001). Raloxifene was shown to activate mechanisms independent of ERE (Mark et al., 2000) in both estrogen receptor dependent and independent manners (Miki et al., 2009).

5.3 ISOLATION OF hMSCs AND VALIDATION OF GH ACTIVITY IN THIS MODEL

Since GH receptors are expressed by bone marrow MSCs (Kassem et al., 1994), I recently started to study the possible GH modulation of MSCs commitment. Having demonstrated the positive modulation of GH signaling by E2 in hOBs, I investigated if the same interaction was present in MSCs. In the present thesis I report some preliminary data of this part of the project.

I isolated human mesenchymal stromal cells from orthopedic waste material obtained after surgery, following a modified version of the protocol of Zhou and colleagues (Zhou et al 2008). As required by the criteria for the use of these cells as an in vitro model, the putative MSCs were tested for the expression of CD73, CD90, CD105 and committed to two lineages, the osteogenic and the adipocytic lineage. The number of positive cells for all the three markers in the 5 different populations analyzed was ca the 46%. This result is in accordance with the view that bone isolated MSCs are a mixture of precursors at different stages and only a little pool is positive for all the CDs markers. Usually the 50% are simply bipotent (Muraglia et al., 2000). Moreover data from our lab (data not shown), demonstrated that the expression profile of CD73, CD90, CD105 is different in the isolated stromal cells compared to that of human osteoblasts. The cells were then tested for their differentiation capability. When cultured in pro-adipogenic and pro-ostegenic media, the isolated cells were able to differentiate both to adipocytes and osteoblasts.

After the characterization of the cells I investigated E2-GH interaction, showing that the enhancement of GH signaling and the decrease in SOCS2 levels mediated by E2 also occurred in hMSCs. Considering that bone marrow adipocytes are increased in number and size in GH deficient dwarf rats (Gevers et al., 2002), and the lack of information about GH action in stromal precursors, I evaluated GH action in the isolated hMSCs, focusing on its possible role in both osteogenesis and adipogenesis. The results showed that long term GH treatment increased significantly the transcription of OPG and

OSX, and of RUNX2, although in this case not significantly, without affecting ADN and C/EBPα. RUNX2 is the master regulator of MSCs commitment towards the osteogenic lineage and it is considered one of the early osteoblastic genes. Therefore the lack of a significant increase after GH could be ascribed to an inadequate time schedule to evaluate it. In fact, after 14 days it is possible that RUNX2 signal could have already returned to basal level, however its action is apparent by the up-regulation of its downstream target genes. This hypothesis is in agreement with the observed up-regulation of the second master regulator of the osteogenic differentiation, OSX, which is downstream to RUNX2. The increase in OSX suggests GH involvement in MSCs commitment towards osteogenesis. OPG has been recently shown to be produced by stromal cells and to be necessary to trigger their osteogenic differentiation (ref). I was not able to evaluate GH effect in MSCs cultivated in osteoMEM, because the differentiating stimulus of the medium overcame GH effect (data not shown). Long term treatment with GH of hMSCs cultivated in adipoMEM induced a reduction in adipogenesis, quantified by a decrease in Oil-Red O accumulation and C/EBPα expression. The divergent data found about the modulation of C/EBPα and ADN by GH in growth and adipogenic media are not in contrast. In fact in growth medium hMSCs did nearly not express ADN and C/EBPα, therefore there is no need to reduce further their expression. On the contrary, in an adipogenic environment, in which the expression of these genes is highly stimulated, GH exerts its pro-osteogenic effect by inhibiting both ADN and C/EBPα expression.

5.4 GROWTH HORMONE EFFECT ON MICRORNAS OF MSCs DIFFERENTIATION AND THE CROSS-TALK WITH E2

A recent and new field of investigation concerns the role of microRNAs (miRNAs) in cellular function. They are short non-coding RNAs, ranging from 18–25 nucleotides, that regulate gene expression by binding to the 3'-UTR (3'

untranslated region) of the target mRNAs and inhibiting gene expression either by promoting degradation of the target mRNAs or avoiding their translation (Erson et al., 2008). MiRNA expression patterns differ in progenitors or fully differentiated cells, suggesting that these miRNAs are highly relevant in MSC lineage committment.

I therefore evaluated if the pro-osteogenic effect of GH on hMSCs involves the regulation of microRNAs. Indeed GH upregulated the two pro-osteogenic miRNAs investigated, namely miR-22 and miR-29c without affecting the pro-adipogenic one, miR-204. MiR-204 is expressed in hMSCs and increases during adipogenic differentiation. Overexpression of miR-204 decreases RUNX2 protein levels whereas the inhibition of the expression significantly elevates RUNX2 protein levels, suggesting that miR-204 acts as an endogenous regulator of RUNX2 in mesenchymal progenitor cells (Huang et al., 2010).
MiR-22 highly and positively correlates with Body Mass Index (BMI) and is involved in BMP7 expression in cartilage (Iliopoulos et al., 2008) In bone it inhibits HDCA6, the transcriptional inhibitor of RUNX2, thus favouring the MSCs osteogenic differentiation while inhibiting the adipogenic one (Huang et al., 2012).
The osteogenic commitment is also promoted by the up-regulation of miR-29 family, while it is negatively affected by their knocking down. Validated targets for miR-29 include several proteins that are inhibitors of osteoblast differentiation. Among the family, miR-29c and miR-29a were identified as target of Wnt, one of the main bone anabolic signals (Kapinas et al., 2009). The preliminary data obtained in this study suggest that the GH pro-osteogenic commitment of hMSCs could involve an up-regulation of specific pro-osteogenic mi-RNAs, without affecting the one related to adipogenesis.
Considering that also in hMSC E2 positively modulates GH intracellular signaling, I investigated if this enhancement could induce an increase in the microRNAs modulated by GH. Surprisingly the results showed that in cells pretreated with E2 there is no enhancing effect of GH on miR-22 and miR-29c.

On the contrary E2 per se reduced miR-22 and miR-29c levels and was able to antagonize GH stimulatory effect. The inhibitory effect of E2 could also be observed on miR-204 levels.

These results suggest that E2 might have an inhibitory effect on MSCs commitment towards adipogenic and/or the osteogenic lineage. The data could appear in contrast with the general idea that E2 drives osteogenesis, while inhibiting adipogenesis (Dang et al., 2002; Zhao et al., 2011). However, these E2 effects were described in MSC cultivated in specific differentiation media. A recent paper has shown that E2 favours hMSCs proliferation when cells are cultivated in normal growth medium and maintains the characteristics of MSCs, including cell surface markers, and osteogenic and adipogenic differentiation capacities (Hong L, 2011). Considering these data and the data I obtained, it seems that E2 could play a different role on the activity of GH on osteogenic cells depending on the stage of differentiation. It might preserve MSCs at an undifferentiated pool and it might promote osteogenic differentiation when cells are already committed. Further researches are needed to evaluate this hypothesis.

5.5 CONCLUSIONS

In conclusion the present study shows a new stimulatory effect of GH pathway by E2 in hOBs. From the data it emerges that the cross-talk between E2 and GH occurs not only systemically, but also in a cell-type-dependent manner.

It was interesting to find that SERM, which are widely used in therapy instead of estrogens for their antagonistic activity in several tissues and agonistic activity in bone, were not able to increase GH pathway as estrogens in hOBs. This aspect should be considered when testing new SERM molecules.

Another interesting aspect is that in bone cells the effect of the interaction of these two hormones is different depending on the maturation stage of the cells. In hOBs there is an enhancement of both the intracellular pathway and of the GH responsive genes IG2, OPN, BSP expression, suggesting that GH and E2 cooperate to activate the bone formation for the correct bone turnover. In hMSCs, there is a positive modulation of the intracellular pathway, however the pro-osteogenic effect of GH involving the upregulation of miR-22 and miR-29c (important in the osteoblastic commitment of MSCs) is inhibited by the effect of E2 *per se* on miRNAs expression. The association GH-E2 thus results in a basal level of miR-22 and miR-29c, suggesting that the cells remain in an undifferentiated state (there are still ongoing experiments to further validate this hypothesis). The cell specificity of the effect of E2 to enhance JAK/STAT pathway activated by GH in hOBs through the inhibition of their negative feed-back exerted by SOCS2 could open new opportunities for pharmacological investigations targeting SOCS2 in order to improve the anabolic action of GH in the bone.

This research provides evidence for a regulatory interaction between estrogens and GH pathway at post receptor level and suggests that estrogens could improve the anabolic action of GH in bone and could influence the responsiveness to GH in GH deficient hypopituitary female.

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