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REVIEW ARTICLE

Follistatin as a potent regulator of bone metabolism

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Abstract

Follistatin is a monomeric glycoprotein, distributed in a wide range of tissues. Recent work has demonstrated that this protein is a pluripotential molecule that has no structural similarity but is functionally associated with members of the transforming growth factor (TGF)-β superfamily, which indicates its wide range of action. Members of the TGF-β superfamily, especially activins and bone morphogenetic proteins are involved in bone metabolism. They play an important role in bone physiology, influencing bone growth, turnover, bone formation and cartilage induction. As follistatin is considered to be the antagonist of the TGF-β superfamily members, it plays an important role in bone metabolism and development.

Keywords: *Follistatin; bones; transforming growth factor-β; activin; bone morphogenetic proteins*

Introduction

Bone metabolic diseases are currently one of the biggest challenges of modern medicine. There is no doubt that susceptibility to bone fractures and bone deterioration increases with age, often due to metabolic changes. However the pathological changes in bone tissue are also observed in young people. The understanding of the structure–function behaviour of bone tissue is very important for both scientists and healthcare providers.

Bone tissue is considered to be a specialized form of connective tissue and the main element of the skeletal tissues. It is composed of cells (osteoblasts, osteoclasts and osteocytes) and an extracellular matrix (ECM) in which fibres are embedded. Bone remodelling is a dynamic, lifelong process in which old bone is removed from the skeleton and new bone is formed. The remodelling process is composed of two distinct stages – resorption and formation – which involve the activity of osteoclasts and osteoblasts (Kaczmarewicz 2000).

Usually, the removal and formation of bone are in balance to maintain skeletal strength and integrity. Bone metabolism is regulated to a large extent by members of transforming growth factor (TGF)-β superfamily. Activin and bone morphogenetic proteins (BMPs) play important roles in bone physiology, influencing bone growth, turnover, bone formation and cartilage induction. Activities of activin and BMPs are regulated by follistatin. Follistatin is a single-chain, monomeric glycoprotein, distributed in a wide range of tissues. It has no structural similarity but is functionally associated with the members of the TGF-β superfamily. As follistatin is considered to be the antagonist of the TGF-β superfamily members, it plays an important role in bone metabolism and development (Lin et al. 2003).

Follistatin

Follistatin is a single-chain, monomeric glycoprotein, distributed in a wide range of tissues. It is considered to be very important in embryogenesis, development and adult life (Phillips & Kretser 1998). Follistatin was first isolated from follicular fluid, when it was found that not only inhibins, the known antagonist of activins, can suppress follicle-stimulating hormone (FSH) secretion (Patel 1998, Phillips & Kretser 1998). It is a glycosylated single-chain protein with no structural similarity but functionally associated with the members of the TGF-β superfamily. The ability to regulate the activity of various members

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of the TGF-β superfamily indicates its wide range of action (Lin et al. 2003). Follistatin is widely distributed throughout adult tissues: it is detected in the pituitary, placenta, ovary, testis, brain, bone marrow, endochondral bone, pancreas and liver (Patel 1998). It has been detected and characterized in a number of mammalian species, including chicken and *Xenopus*. Follistatin protein is highly conserved among species with 97% amino acid homology between human and mouse (Lin et al. 2003). The human follistatin gene localizes to chromosome 5q11.2, is relatively small (~6 kb) and consists of six exons. The first encodes a putative signal sequence; it is followed by four exons encoding four domains and the last exon encodes for the 27 extra amino acids at the carboxyl terminal of the 344-residue precursor. An alternative mRNA splicing occurs at the 3′-terminal of the gene, between exons 5 and 6 and generates the precursor follistatin 317 mRNA (pre-FS-317 mRNA) and the precursor follistatin-344 mRNA (pre-FS-344 mRNA) (Figure 1). Cleavage of the signal peptide (29 amino acids) leads to formation of mature follistatin isoforms: FS-288 (containing 288 amino acid residues) and FS-315 (containing 315 amino acid residues) (Figure 1) (Lin et al. 2003, Patel 1998, Phillips & Kretser 1998, Wankell et al. 2001). There are reports that confirm that FS-315 further undergoes enzymatic cleavage to a shorter intermediate form consisting of 300–303 residues (Welt et al. 2002).

Distribution of these two isoforms of follistatin is not well characterized; however FS-315 is likely to be the circulating form, because of the higher affinity of FS-288 for cell surface heparin sulfate proteoglycans (Bilezikjian et al. 2003, Lin et al. 2003, Phillips & Kretser 1998, Risbridger et al. 2001). The FS-315 variant is considered to be predominant, because mRNA producing FS-288 accounts for less than 5% of the follistatin mRNA encoded (Patel 1998, Phillips & Kretser 1998, Wankell et al. 2001). However FS-288 possesses higher activity because of its greater affinity for cell surface proteoglycans (Wankell et al. 2001). This occurs through a consensus of heparin sulfate binding sites, which is the cluster of repeated basic (Lys, Arg) residues. The predominant circulating form of follistatin does not interact with the heparin-binding site probably because it is masked by the of C-terminal extension of this molecule (Phillips & Kretser 1998, Welt et al. 2002). FS-288 is considered to be the predominant form present in human follicular fluid, whereas the main form in serum is FS-315 (Lin et al. 2003).

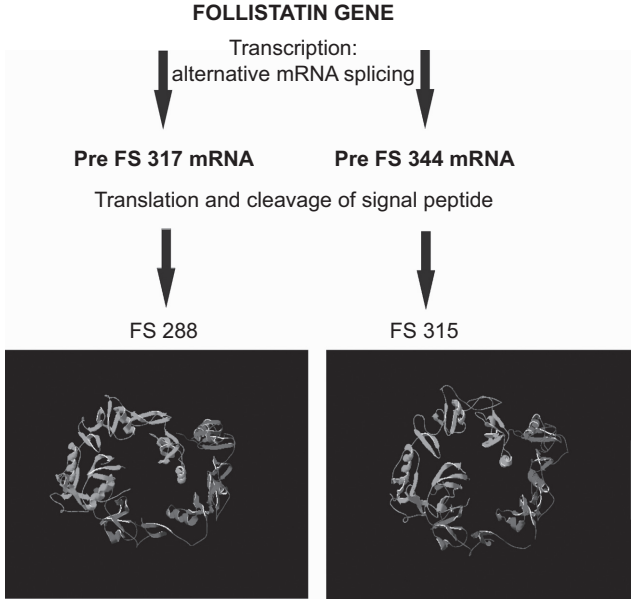

Figure 1. Schematic representation of the follistatin gene, alternative splicing and protein processing.

Characterization of the TGF-β superfamily

The TGF-β superfamily of growth factors comprises more than 30 structurally related mammalian proteins that have diverse functions during embryonic development and adult tissue homeostasis (Lin et al. 2003). They control various physiological processes that are important for development of the organism, reproduction and health. It was found that during embryogenesis, they act as morphogens establishing cell fate asymmetries along dorsal/central and anterior/posterior axes, whereas in the adult they regulate reproductive functions, the immune system, wound healing and tissue regeneration. The TGF-β superfamily can be divided into three subfamilies: the TGF-βs, activins/inhibins and BMPs, although some TGF-β superfamily members may fall outside these groups (Lin et al. 2003, Thompson et al. 2005). Members of this superfamily are synthesized as large precursor proteins, composed of an amino-terminal signal sequence, pro-domain and mature protein (carboxyl-terminal domain). The signalling parts of the molecules are the hetero- or homodimers of the highly conserved carboxy-terminal domain, mostly containing seven conserved cysteine residues. Six of these cysteines form a cystine knot, and the seventh forms an additional disulfide bond participating in dimerization with a second monomer. The presence of the seven conserved cysteines, involved in folding of the molecule, is considered to be the common feature of molecules belonging to the TGF-β superfamily. Common mechanisms of signal transduction are observed among the members of the TGF-β superfamily. They bind to transmembrane serine–threonine kinase receptors, leading to assembly of a receptor complex and phosphorylation of the Smad family proteins. Next, the Smads move into the nucleus and bind DNA, leading to the recruitment of transcriptional co-activators or co-repressors to control the expression of target genes. Two groups of transmembrane serine–threonine kinase receptors are distinguished and designated type I and

type II receptors. Generally each ligand has its own specific receptor and after it binds, the activated type I and type II receptor complex is formed. The type II receptor phosphorylates the type I receptor, which in turn phosphorylates the Smad proteins that transmit the signal. Smad proteins are classified into the following subgroups: receptor-mediated Smads (R-Smads), a common partner Smad (co-Smad) and the inhibitory Smads (I-Smads) (Lin et al. 2003) (Figure 2).

Transforming growth factor-β

TGF-β is a pleiotropic growth factor that regulates cell differentiation, cell division, immune function and ECM production. Three mammalian homodimeric TGF-β isoforms (TGF-β1, -β2 and -β3) can be distinguished (Olakowski 2007). Each isoform is coded by a distinct gene, whose expression is tissue specific (Olakowski 2007). The TGF-β1 isoform is expressed in endothelial cells as well as in hematopoietic cells of connective tissue. TGF-β2 is detected in nerve cells and epithelium, whereas TGF-β3 is present in mesenchymal cells (Epstein 2005, Olakowski 2007). TGF-β is synthesized in the latent form, associated with latent TGF-β-binding protein (LTBP). Then under the action of plasmin or thrombospondin, the created complex is proteolytically cleaved, leading to the production of active forms of TGF-β in cells (Epstein 2005). The mature polypeptides of these isoforms share

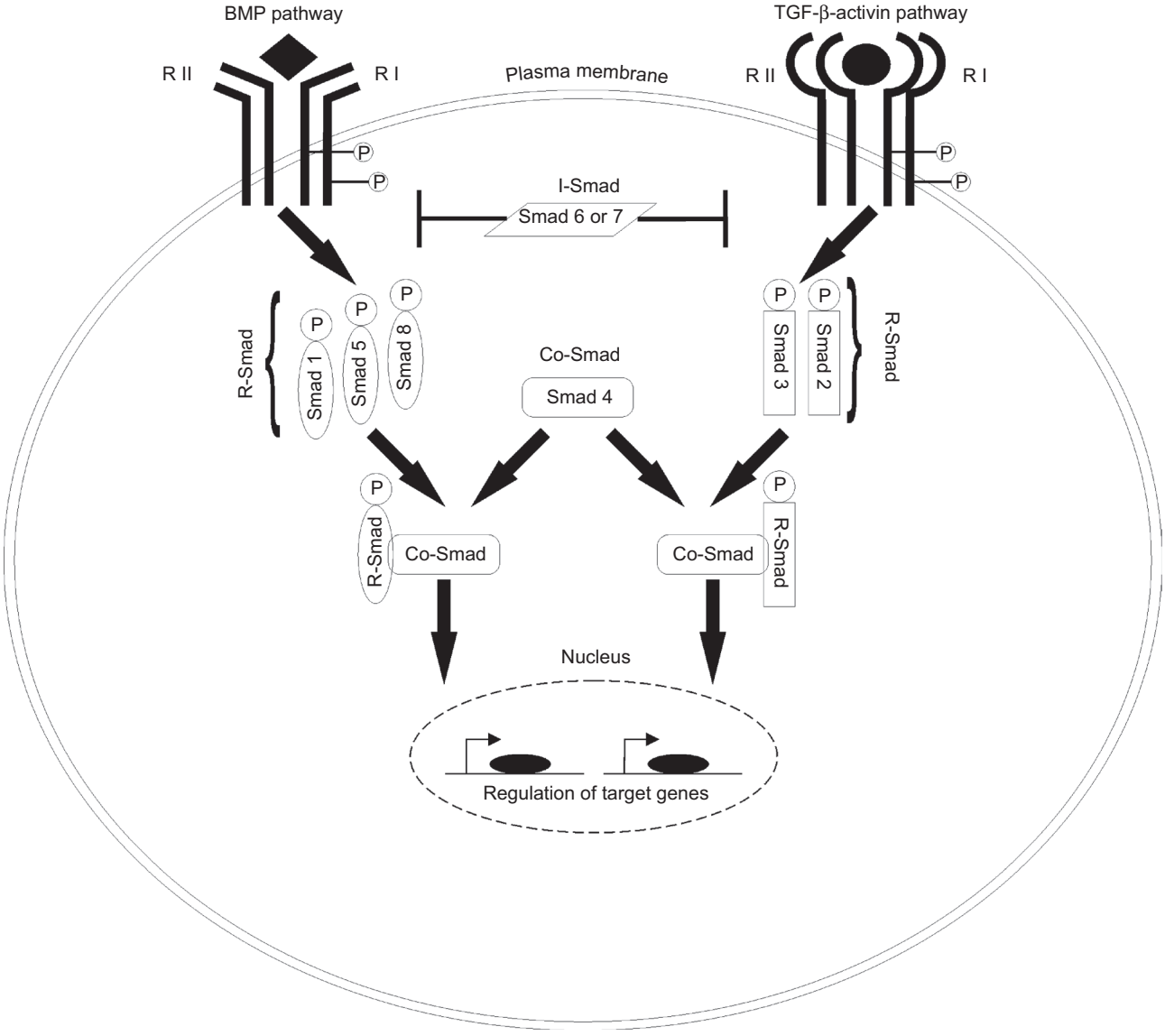

Figure 2. The basic transforming growth factor (TGF)-β superfamily members SMAD pathway. After formation of a ligand–receptor complex, receptor type I (phosphorylated by serine–threonine kinase type II), phosphorylates R-Smad (Smad 1, 5, 8 for bone morphogenetic proteins (BMPs)) and Smad 2 and 3 for TGF-β/activin. The further connection of R-Smad with co-Smad (Smad 4) creates the complex that moves into the nucleus and regulates the transcription of target genes.

80% sequence homology with each other and have similar biological activities (Steiner 1995). Biological activity of TGF-β is mediated by heteromeric receptor complexes consisting of a type I and type II transmembrane serine–threonine kinase receptors. Binding to the receptor leads to the phosphorylation of the intracellular receptor-associated proteins R-Smad (Smad 2 and Smad 3), which in conjunction with co-Smad (Smad 4) regulate transcriptional responses (Lin et al. 2003). TGF-β is found in a wide variety of tissues and in all primary embryonic germ layers. It possesses complex multifunctional biological properties, and it can be a cell growth stimulator or inhibitor, depending on cell type and cell differentiation state. Moreover it is able to stimulate angiogenesis and ECM formation, whereas it suppresses the immune system. Furthermore it regulates cellular differentiation as well as cell adhesion. For that reason TGF-β plays important role in embryogenesis, wound healing and other cellular processes (Steiner 1995).

Inhibins and activins

Inhibins and activins are dimeric glycoproteins, formed by two or three different subunits (α, βA and βB). Inhibins are composed of one of the β-subunits dimerized with a common α-subunit (α:βA and α:βB designated as inhibin A (Inh A) and inhibin B (Inh B), respectively) and inhibin production is restricted to steroidogenic tissues and the hypophysis (Kretser et al. 1999, Patel 1998, Phillips & Kretser 1998, Vänttinen et al. 2002). Activins are dimeric proteins composed of two inhibin β proteins, encoded by five distinct inhibin β subunit genes, designated as βA, βB, βC, βD and βE. The activin A (the inhibin βA dimer) and activin B (the inhibin βB dimer) are biologically active forms (Kretser et al. 1999, Patel 1998, Phillips & Kretser 1998, Vänttinen et al. 2002, Wang et al. 1999), and their β subunits are disulfide-linked (Inoue et al. 1994). Biological activity of activins is mediated by heteromeric receptor complexes consisting of a type I (ActRIA or ActRIB) and type II (ActRIIA and ActRIIB) transmembrane serine–threonine kinase receptors (Kretser et al. 1999, Krneta et al. 2006, Wankell et al. 2001). Binding to the receptor leads to the phosphorylation of the intracellular receptor-associated proteins R-Smad (Smad 2 and Smad 3), which in conjunction with co-Smad (Smad 4) regulate transcriptional responses (Dow et al. 2005). Activins have multiple effects on many cell types, where they are able to stimulate or inhibit cellular proliferation (Ageta et al. 2008, Krneta et al. 2006, Vänttinen et al. 2002, Wang et al. 1999). Activins are known to contribute to the dynamic changes of the level of FSH that are observed throughout the reproductive cycle (Dalkin et al. 1996). FSH is a glycoprotein secreted by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH), released by the hypothalamus and gonadal steroids. Activin is able to increase the level of FSH-β mRNA and its secretion, whereas inhibins counteract this activity (Kaiser & Chin 1993, Welt et al. 2002). It was demonstrated that in the absence of endogenous activin, the synthesis of FSH-β is nearly undetectable. Moreover, the activin treatment resulted in upregulation of FSH-β mRNA by up to 55-fold in comparison to 3-fold stimulation by pulsatile GnRH. Folliculostellate cells produce follistatin, which is considered to be the intrapituitary, paracrine regulator of FSH secretion. Inhibins and follistatin are able to act together as extracellular regulators of activin action in order to regulate timing, duration and amplitude of FSH secretion (Welt et al. 2002).

There are many potential pathways in which follistatin regulates FSH synthesis and secretion, although these are mediated through neutralization of activin actions, rather than by influencing the activin synthesis in the pituitary. Follistatin is able to suppress the release of FSH induced by activins and GnRH, whereas it has no influence on the level of luteinizing hormone (Kaiser & Chin 1993, Phillips & Kretser 1998). Both follistatin and inhibin are able to modulate the activity of activin. Follistatin prevents activin from interacting with its receptor whereas inhibins compete for binding to ActRII, preventing recruitment of the activin type I receptor (Eijken et al. 2007). It has been reported that both activins and inhibins share the same binding site for this type of receptor (β-subunit). Affinity of inhibins for ActRII is about tenfold lower than that of activin, and in some tissues inhibins do not antagonize the activity of activins. However the co-receptor of inhibin called betaglycan was identified. It binds inhibins with high affinity, increasing about 30-fold the affinity toward ActRII in cells coexpressing both ActRII and betaglycan (Harrison et al. 2005). Formation of the activin–follistatin complex is almost irreversible and consists of one activin dimer and two follistatin molecules, i.e. one follistatin molecule binds to one activin subunit (Phillips & Kretser 1998). In contrast, inhibin containing one β-subunit has only one binding site for follistatin, implying that follistatin binds to activin and inhibin through the common β subunits. It is unclear whether follistatin can prevent the binding of inhibin to the activin receptor (Lin et al. 2003); however the affinity of follistatin for activin is high, the K_d is estimated as 50–900 pM, which is very similar to the affinity of activins for their receptors (McPherson et al. 1999, Phillips & Kretser 1998). This explains why follistatin is considered to be the potent modulator of the action of activins (Lin et al. 2003). The affinities of known isoforms of follistatin towards activins are almost identical (Patel 1998). The mechanism by which follistatin neutralizes activin depends on the follistatin isoform that antagonizes its action. The interaction of surface-bound follistatin (FS-288) leads to rapid endocytosis by lysosomal enzymes; for that reason it is an almost irretrievable breakdown pathway. As the affinity

of FS-315 to surface proteoglycans is low, the captured activin is inactivated and cannot bind to its receptor, but the complex is not endocytosed (Phillips & Kretser 1998). Activins are synthesized in a wide variety of tissues and cell types and the pattern of their action is consistent with an autocrine/paracrine mechanism, rather than endocrine, because circulating activin appears to be bound almost irreversibly to follistatin. In order to act in an endocrine way activin would have to be released from follistatin by some proteolytic cleavage. Such a mechanism has been identified for BMPs, in which bioactive BMP bound to its bioactive protein chordin is released by metalloproteinase, toloid. Follistatin action is also largely autocrine/paracrine in nature. This assumption was proved by several studies employing a heterologous and homologous follistatin radioimmunoassay (RIA). Heterologous follistatin RIA, using antibovine follistatin antibody, found similar serum follistatin concentration in both sexes, fertile or not and regardless of menstrual cycle stage. The results indicated that gonadal follistatin production has no obvious influence on circulating follistatin levels, and thus failed to support an endocrine role for circulating follistatin in regulation of pituitary FSH release. Another assay concerning homologous follistatin RIA also suggested that follistatin is primarily an autocrine/paracrine modulator of activin action rather than a circulating endocrine hormone. A homologous follistatin RIA was developed with a mouse polyclonal antiserum to human recombinant FS-288. Neither activin-A nor inhibin-A had any effect on follistatin quantitation in this study. Likewise in a previous RIA assay similar follistatin concentrations were detected in both females and males, with no difference across the menstrual cycle. Moreover no differences were found in normally cycling women in comparison to postmenopausal individuals. Although high follistatin concentrations are achieved in developing human follicles, the serum and follicular follistatin concentrations were found to be biochemically distinct (Welt et al. 2002).

Circulating follistatin is bound to activin and may act to facilitate the clearance of activin and/or prevent its diffusion from activin's site of action (Welt et al. 2002). Follistatin together with activin creates a pleiotropic growth factor system that controls in an autocrine or paracrine manner the proliferation, differentiation and apoptosis of many cell types (Krneta et al. 2006) (Figure 3).

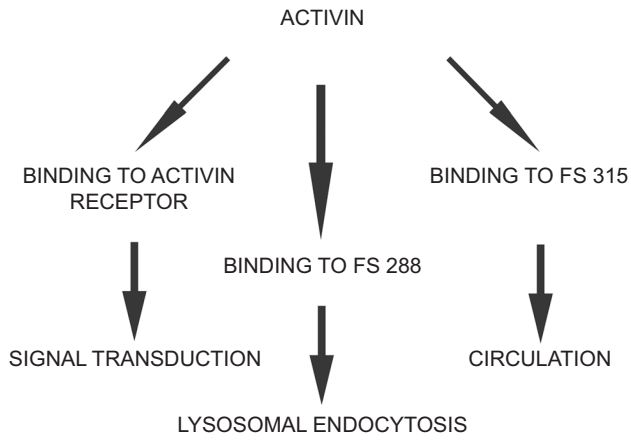

Figure 3. Activin directing pathways.

Bone morphogenetic proteins

Follistatin next to gremlin, chordin and noggin is considered to be an antagonist of BMPs (Tardif et al. 2004, Wan & Cao 2005). It exerts its action by forming a complex with BMPs and preventing their proper binding to the receptors. All BMPs, also called osteogenic proteins (OPs), are secreted as a precursor protein with a hydrophobic stretch containing about 50–100 amino acids (Xiao et al. 2007). They are the signalling molecules whose biological activity is related only to the dimeric form; both homodimers and heterodimers are considered to be active (Kochanowska et al. 2007). About 20 different proteins are now considered to belong to the family of BMPs, which can be divided into the following groups: BMP-2/4, BMP-3, BMP-7, including BMP-5-11, and BMP-12-15 (Epstein 2005). BMPs as members of the TGF-β superfamily exert their action by binding to type I (BMPRI) and type II (BMPRII) serine/threonine kinase receptors. Among the BMPRI group BMPR-IA (ALK-3), BMPR-IB (ALK-6) and ActRI-A (ALK-2) receptor types can be distinguished. There are also three types of type II receptors: BMPRII and type I and II B activin receptors (ActRII and ActRII-B) (Wan & Cao 2005, Xiao et al. 2007). BMPs bind with weak affinity to type I or type II receptors alone, and with high affinity to a heteromeric complex of the two receptor types, so both type I and type II receptors are required for BMP signalling. It was found that the affinity of BMPRI for ligand binding is higher than that of BMPRII, so it is plausible that the ligand is initially bound to BMPRI and then this receptor recruits BMPRII into the ligand–receptor complex. The next step is that activated type II receptor kinase phosphorylates type I receptor, which initiates the intracellular signalling by the phosphorylation of downstream components, including Smads, known as nuclear effector proteins. The complex of Smad proteins is formed and then it is translocated to the cell nucleus and after the interaction with transcriptional factors it activates the transcription of target genes (Vukicevic & Sampath 2004). The BMP-specific R-Smads include Smad 1, Smad 5 and Smad 8. They form heteromeric complexes with the co-Smad (Smad 4) and translocate into the nucleus (Lin et al. 2003, Vukicevic & Sampath 2004). Furthermore it was found that Smad 1 or Smad 5 synergizes with Smad 4 to promote chondrocyte differentiation from chondroprogenitor cells. BMPR-IA is

more widely expressed than BMPR-IB in various tissues; however BMPR-IB is considered to be the only receptor type expressed within cartilage. Expression of ALK-2 is observed in osteoblasts and chondrocytes (Wan & Cao 2005). As their name indicates BMPs are able to induce ectopic cartilage and bone formation, a process that mimics embryonic endochondral bone formation (Xiao et al. 2007). BMPs play an important role in bone physiology by influencing bone growth, bone turnover, bone healing and cartilage formation (Kochanowska et al. 2007). They are the important factors that regulate the processes of chondrogenesis and skeletogenesis during normal embryonic development (Xiao et al. 2007). Apart from bone forming activity BMPs are potent chondrogenic morphogens and are capable of inducing differentiation of mesenchymal stem cells into the cell lineage of hyaline cartilage (Vukicevic & Sampath 2004). The BMPs with the greatest osteogenic capacity include: BMP-2, -4, -5, -6, -7 and -9. It was found that in bone formation BMP-2 together with BMP-7 promotes the expression of critical transcription factors Runx2 and Osterix in mesenchymal stem cells, thereby committing them and directing them in osteoblast differentiation (Xiao et al. 2007). BMPs induce new bone formation because they are able to promote the recruitment and growth of osteoblast progenitor cells and maintain their expression. Hence, providing the optimal dose of BMP in the circulation may help to trigger osteogenic responses to restore the loss of bone mass in osteoporosis and other metabolic bone diseases (Vukicevic & Sampath 2004). The important role of BMPs was demonstrated by experiments employing genetically engineered mice. Mice deficient in BMP-2 die between days 7 and 10 of gestation of cardiac defects before bone formation; furthermore BMP-7-deficient mice exhibit skeletal alterations in the rib cage, hind limbs and skull. Also BMP-5- and BMP-6-knockout mice present skeletal defects. The exception is BMP-3, because it plays an opposite role to BMP-4 and inhibits BMP-2-induced osteogenic differentiation, which results in increased bone density. Additionally BMPs are also widely distributed in non-skeletal tissues such as nerve, gastrointestinal tract, kidney, heart and lungs. Neuro-, cardio- and reno-protective actions of BMPs are known, which indicates their therapeutic use (Xiao et al. 2007). They affect epithelial cells, monocytes and neuronal cells. Furthermore BMPs serve as inductive signals for overall tissue development during embryogenesis (Vukicevic & Sampath 2004). There is strong evidence indicating the important role of BMPs in regulation of stem cell properties. In embryonic stem cells (ESCs), BMP signalling is required for self-renewal of ESCs but this depends on its ability to block neural differentiation. Furthermore in mesenchymal stem cells, BMP signalling induces osteoblastic differentiation by interaction with BMPR-1B, but it inhibits osteoblastic differentiation through BMPR-1A (Xiao et al. 2007). Follistatin modulates the activity of BMPs by blocking the signal transduction and this makes the interaction with its specific receptors impossible. It binds to BMP receptors and BMPs, forming a trimeric complex. Follistatin is able to bind BMP-2, BMP-4 and BMP-7, although the affinity for BMPs is lower than that for activin. Follistatin inhibits BMP signalling in a different manner compared with other mentioned antagonists (Tardif et al. 2004). However there are visible differences between follistatin and other BMP-binding proteins noggin and chordin, which prevent BMPs from interacting with its receptor. Furthermore follistatin binds to BMPs with weaker affinity compared with chordin and noggin (Amthor et al. 2002).

TGF-β superfamily members and follistatin system in bone tissue

Bone is composed of highly structured ECM-containing osteoblasts, osteocytes and osteoclasts. Osteoblasts, of mesenchymal origin, synthesize and secrete bone matrix, which undergoes mineralization. Once embedded in bone matrix, osteoblasts become differentiated into osteocytes, whereas the role of osteoclasts, which are of haematopoietic lineage, is concentrated around bone resorption. Osteoblasts together with osteoclasts participate in bone remodelling that maintains proper bone structure and integrity. Defects in this process can lead to progressive changes in bone mass and quality. Members of the TGF-β superfamily have attracted attention for their involvement in bone metabolism.

It is known that TGF-β is locally produced in bone tissue and bone is considered to be the largest reservoir of TGF-β in the organism. It is accumulated in bone matrix and released from bone in the process of bone resorption. Both osteoblasts and osteoclasts secrete TGF-β and all TGF-β isoforms (TGF-β1, -β2 and β3) are present in latent form within the bone matrix. Deposited in bone matrix, TGF-β isoforms are released during bone resorption and then are activated by osteoclasts. This activation leads to the induction of nearby osteoblastic differentiation. TGF-β is considered to be a physiological regulator of osteoblast differentiation and a key mediator of the coupling of osteoblast differentiation to osteoclastic bone resorption required for skeletal homeostasis (Filvaroff et al. 1999). Furthermore, independently from the mitogenic action, TGF-β enhances the production of type I collagen by bone cells. Type I collagen constitutes about 90% of the organic component of bone matrix and its macromolecular organization is important in proper bone calcification. Experiments performed on osteoblast cultures demonstrated increased collagen synthesis after TGF-β treatment. Furthermore, many studies indicate that TGF-β is able to reduce the activity or production of

a variety of collagen and bone matrix-specific proteinases and promote the synthesis of proteinase inhibitors. It was found that the increase in association of secreted collagen with other matrix components under the influence of TGF-β contributes to the maintenance of bone integrity. TGF-β, together with other osteogenic growth factors or those released at wound sites, contributes to growth, remodelling and repair of bone tissue (Centrella et al. 1992). Next to TGF-β, BMPs play important roles in bone physiology, influencing bone growth, turnover, bone formation and cartilage induction (Kochanowska et al. 2007). Because of the finding that TGF-β and BMPs are involved in bone metabolism, it had been predicted that activin, as a member of the TGF-β superfamily, would also participate in this process (Sakai & Eto 2001).

Beside the activity of inhibins on the reproductive axis, they play a crucial role in regulation of bone turnover and bone quality (Perrien et al. 2006, 2007). *In vitro* studies demonstrated that inhibins have the ability to inhibit both osteoblast and osteoclast development in murine bone marrow cultures (Perrien et al. 2006). Furthermore other experiments proved that both Inh A and Inh B suppressed osteoblastogenesis from mesenchymal stem cells, together with late-stage osteoblast differentiation and mineralization. InhA and inhB also completely blocked receptor activator of nuclear receptor κB-ligand (RANK-L)-induced osteoclastogenesis from peripheral mononuclear cells (Ebeling 2006). These studies confirm that inhibins are direct regulators of bone cell differentiation and bone turnover (Perrien et al. 2006). Because the previous demonstration showed the direct influence of inhibin on osteoblast and osteoclasts development Perrien et al. (2007), tested whether InhA regulates bone mass *in vivo*, using a transgenic mice model of inducible human Inh A expression. *In vivo* experiments performed on transgenic mice, expressing human InhA, demonstrated that Inh A is a nonsteroidal, gonadal regulator of bone mass, that increases bone formation with little or no effect on osteoclasts or bone resorption. InhA is the endocrine hormone, whose stimulatory action on bone tissue significantly differs from those of other TGF-β superfamily members whose effects on skeleton are associated with their local production in bone. It was demonstrated that the continuous InhA exposure leads to the increase of total body bone mineral density, bone volume and strength, improving biomechanical properties of bones of intact adult mice and prevents the loss of bone mineral density, bone volume and strength associated with gonadectomy, as in both genders, gonadal function is essential for the maintenance of bone quality (Perrien et al. 2007). Other studies showed the inverse correlation between both Inh A and Inh B level and the markers of bone formation and resorption in premenopausal women. However in postmenopausal women only Inh A is inversely correlated with bone formation markers. This is consistent with the idea that inhibins rather than FSH are able to suppress bone turnover by suppressing osteoblastogenesis, thereby reducing support for osteoclastogenesis (Perrien et al. 2006).

Both *in vivo* and *in vitro* studies show that inhibins exert a bimodal effect on the regulation of bone metabolism. It was found that the normal physiological exposure to inhibins suppresses bone turnover, whereas the long-term continuous exposure to inhibins is anabolic. The stimulatory effect of InhA on bone tissue is associated with the increase of mature osteoblast activity and differentiation. The significant increase of bone mass induced by Inh A is thought to be independent of changes in osteoclast number or function (Perrien et al. 2007).

Activin A (βA homodimer) is the only activin form that is known to be present in bone as well as in cartilage. Several studies demonstrated its activity in bone tissue formation (Ikenoue et al. 1999). It was found that activin A mRNA is produced in locally in bone marrow and similar to TGFβ and BMPs, activin βA subunit is abundantly localized in bone matrix (Gaddy-Kurten et al. 2002). Activin A is synthesized both by osteoblasts and osteoclasts. *In vitro* and *in vivo* studies have shown the involvement of activin A in bone formation, although, there are also reports presenting an inhibitory activity of activin A on osteoblasts differentiation (Eijken et al. 2007).

In vitro, activin A is able to stimulate the proliferation of osteoblastic cells and enhances matrix secretion by these cells (Fuller et al. 2000). It was reported that activin A exerts a mitogenic effect on murine osteoblast-like MC3T3-E1 cells; moreover, these cells are able to produce follistatin at the mRNA and protein level (Inoue et al. 1994). It was found that, in MC3T3-E1 cells containing a high number of activin A binding sites, the mitogenic effect of activin on cell replication and inhibitory effect of expression of alkaline phosphatase was observed (Hashimoto et al. 1992).

In vivo experiments showed that periosteal injection of activin A stimulates bone formation, and ectopic bone formation induced by BMP, as well as its local administration promotes fracture healing (Fuller et al. 2000). Moreover local administration of activin A significantly increased the periosteal bone matrix thickness in newborn rat parietal bone, as well as enhanced the non-cartilaginous ectopic bone formation stimulated by BMP-2 (Gaddy-Kurten et al. 2002). Furthermore it was found that embryonic bone tissue expresses mRNA for the βA subunit of activin and it is able to stimulate proliferation and collagen synthesis in osteoblastic cells. This stimulatory activity on ectopic bone formation is inhibited by follistatin, as follistatin inhibits the biological activity of activins (Sakai & Eto 2001). Similarly during endochondral bone development, the stimulatory

actions of activin are regulated by changes in follistatin expression during the transition from cartilage to bone. It was found that follistatin mRNA as well as follistatin protein has been detected in both osteoblasts and osteocytes in developing mouse mandible and in fracture callus. Recent studies in rats demonstrated that administration of exogenous systemic activin A in low doses counteracts the ovariectomy-induced vertebral bone loss and favours bone tissue formation (Gaddy-Kurten et al. 2002). Furthermore it appeared that the osteogenic activity of activin is different from that of TGF-β and BMP (Sakai & Eto 2001). Sakai & Eto (2001) showed that injection of activin onto the periosteum of newborn rat calvarium resulted in an increase in the thickness of the bone, whereas the local injection of TGF-β stimulates the proliferation of periosteal layer cells. Probably these three members of the TGF-β superfamily cooperate with each other in the regulation of bone formation during fracture healing (Sakai & Eto 2001).

However another report indicated the inhibitory effect of activin on osteoblasts differentiation. Ikenoue et al. (1999) demonstrated the inhibitory effect of activin A on differentiation of fetal rat calvarial (FRC) cells in early phase culture. It was found that addition of activin A was able to inhibit gene expression of matrix proteins (type I procollagen, osteopontin, osteonectin and alkaline phosphatase). These proteins, known to be osteoblast differentiation markers are as necessary for bone nodule formation. Although activin A was able to inhibit the expression of the mentioned bone-related proteins, the increase of expression of osteocalcin was observed in FRC cell cultures. This was because osteocalcin was the marker of terminal osteoblasts' differentiation or was synthesized by mature osteoblasts, whereas activin A receptor was expressed in early cell culture phase. Activin A inhibited early differentiation of FRC cells, but did not exert any effect on cell proliferation, suggesting that this cytokine may hold the premature state of the cells in a proliferating condition (Ikenoue et al. 1999). Similar results were observed in murine MC3T3-E1 cells, where Hashimoto et al. (1992) also demonstrated the inhibitory effect of activin A on the expression of alkaline phosphatase (Figure 4).

Activin A, similarly to TGF-β, is involved in the coupling between bone formation and resorption, because of its osteogenic activities and its release coupled to bone resorption (Sakai & Eto 2001). It has been reported that activin A enhances osteoclast formation in organ culture and activin A gene expression and secretion by bone marrow stromal cells is upregulated by bone-resorbing cytokines: tumour necrosis factor (TNF)-α and interleukin (IL)-1α. Furthermore it was found that activin A strongly synergizes with RANKL, a receptor activator of NFκB that is necessary in osteoclastogenesis (Fuller et al. 2000). As activin and follistatin are present in bone matrix, the activin–follistatin system is involved in bone modelling as well as in bone remodelling (Funaba et al. 1996). Activin also has the potential

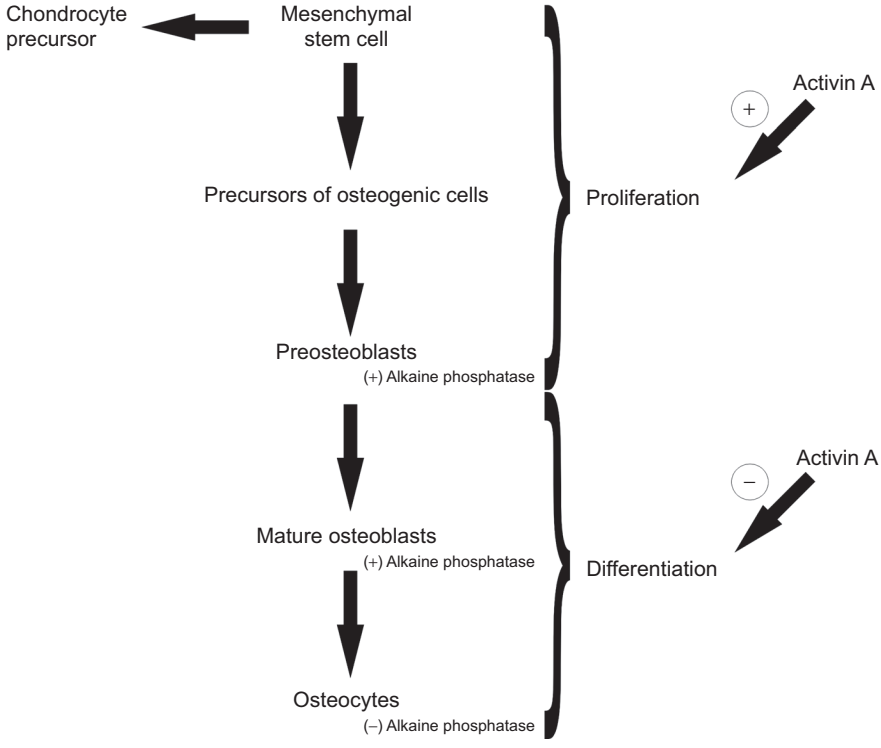

Figure 4. The influence of activin A on proliferation and differentiation of osteoblasts (Ostrowski 1995).

to control the process of mineralization of ECM. Bone quality is mainly determined by the protein composition and mineralization of ECM. As bone-forming cells, osteoblasts regulate bone quality, undergoing complex differentiation processes. During differentiation and bone formation, osteoblasts produce a complex ECM, which eventually starts to mineralize. The process of mineralization requires the precipitation and attachment of calcium phosphate crystals to ECM. Activin A controls the function of osteoblasts and their differentiation; furthermore it is able to inhibit matrix mineralization through altered ECM composition and maturation. Activin A strongly inhibits the mineralization potential of ECM. Gene expression studies showed that ECM genes that are upregulated by activin A signalling (low mineralization) may act negatively on mineralization, whereas ECM genes that are downregulated may act positively (Eijken et al. 2007). Activin A action is controlled by follistatin and the activin A–follistatin mechanism controls the extent of mineralization. This is very important because over-mineralization of bone tissue can result in brittle bones that are too stiff and unable to reform during loading. Also TGF-β can inhibit matrix mineralization in osteoblast cultures; however TGF-β is activated only during bone resorption by osteoclasts. In contrast, activin A and follistatin are able to control the mineralization process by a mechanism that is independent of a direct coupling to resorption. Activin A signalling is a potent regulator of bone matrix formation and mineralization and thereby is an important mechanism in the control of bone quality (Eijken et al. 2007).

Moreover, members of the TGF-β superfamily are also employed in the process of endochondral bone formation. It comprises a series of events consisting of differentiation of chondroblasts, cartilage hypertrophy, vascular invasion, osteogenesis and marrow differentiation. The process of chondrogenesis is mainly regulated by Sox9, a transcription factor of the SRY (sex-determined region on the Y chromosome) family. TGF-β superfamily members together with Sox9 regulate the process of endochondral bone formation. It was found that BMP signalling regulates *Sox9* expression, because inactivation of BMPR-1A and BMPR-1B in mice resulted in a lack of *Sox9* expression. Moreover it was observed that also TGF-β controls *Sox9* expression during chondrogenesis (Kawakami et al. 2006). Together with members of the TGF-β superfamily, follistatin is involved in cartilage and bone development. It was found that a greater expression of follistatin was detected at the initial stages of chondrogenesis and osteogenesis. So both chondrocytes and osteoblasts produce follistatin, but only during the proliferating states, and it is no longer expressed in cells that have stopped proliferating. Follistatin produced by proliferating osteoblasts is accumulated in bone matrix during endochondral bone development. It may be bound to proteoglycans in bone matrix, because of its association with heparin and heparin sulfate (Funaba et al. 1996).

TGF-β and activins as essential cytokines in osteoporosis and metastatic bone disease

Osteoporosis is considered to be a disease, characterized by low bone mass and structural deterioration of bone tissue, which leads to bone fragility and increased susceptibility to fractures (van Lenthe et al. 2008). Correct bone structure and integrity are maintained through the proper bone remodelling process and coordinated by relative activities of both osteoblasts and osteoclasts (Filvaroff et al. 1999). Deregulation of microanatomical coupling between both types of cells leads to the pathological loss of bone mass seen in osteoporosis or other metabolic bone diseases (Erlebacher et al. 1998).

Proteins from the TGF-β superfamily play an important role in bone remodelling processes. Both osteoblasts and osteoclasts secrete TGF-β, and all TGF-β isoforms are present in the bone matrix. TGF-β is considered to be the physiological regulator of differentiation of osteoblasts, as well as a key mediator of the coupling of osteoblast differentiation to osteoclastic bone resorption required for skeletal homeostatis (Erlebacher et al. 1998). In order to characterize the regulation of the function of osteoblasts and osteoclasts by TGF-β during the bone remodelling process, Erlebacher et al. (1998) generated transgenic mice, overexpressing TGF-β2 from the osteoblast-specific osteocalcin promoter. Transgenic mice demonstrated the intensive age-dependent loss of bone mass similar to that seen in osteoporosis and hyperparathyroidism, as well as defects in skeletal development and growth. In this experimental model, an increase of bone matrix-embedded osteocytes, an increase in the rate of osteoblastic bone formation as well as increase of osteoclastic bone resorption was observed. It was reported that TGF-β-induced positive regulation of osteocyte density, resulted from direct, autocrine effect on osteoblasts and occurs even at endogenous levels of TGF-β expression. Furthermore the increase in the rate of osteoblastic bone formation was not associated with the direct response of osteoblasts to TGF-β, but was the secondary consequence of increased bone resorption. Accordingly, as increased TGF-β activity may be involved in increased osteocyte density, it may contribute to development of several bone metabolic diseases such as osteoporosis, hyperparathyroidism and osteogenesis imperfecta. So deregulation of skeletal TGF-β expression, activation and responsiveness in humans may have crucial physiological consequence, leading to pathological bone-remodelling states (Erlebacher et al. 1998). Because TGF-β has been postulated to play an important role in the control of bone density by regulation of the balance between bone

matrix deposition by osteoblasts and its resorption by osteoblasts, Grainger et al. (1999) examined if the serum levels of TGF-β isoforms might be useful as a clinical tool in osteoporosis. The concentration of each TGF-β isoform (TGF-β1, TGF-β2 and TGF-β3) was determined with the application of enzyme-linked immunosorbent assay (ELISA) kits. The serum of women suffering from osteoporosis versus healthy women was examined. It was found that serum levels of both TGF-β1 and TGF-β2 were very similar, whereas the concentration of TGF-β3 was significantly raised in osteoporotic women. The results indicate that the level of TGF-β3 can be treated as a potential biological marker of osteoporosis.

Activin A is present in bone matrix, and its release associated with bone resorption and its osteogenic activities indicate a possible involvement of activin in the coupling between bone resorption and formation. Considering that one of the major causes of bone loss in osteoporosis is uncoupling between bone formation and resorption processes would be of great interest to know if activin treatment prevents bone loss in osteoporosis (Sakai & Eto 2001). In order to determine the effects of exogenous activin on bone mass Sakai and Eto (2001) performed the experiment on aged ovariectomized (OVX) rats. They found that vertebral bone mineral density (BMD) and bone mineral content (BMC) were visibly increased in the rats treated with activin, indicating its osteogenic activity. Moreover biomechanical tests resulted in a significant increase in the maximum load and stiffness of vertebral bodies in the group of activin-treated rats. Furthermore the positive correlation between BMD of vertebral bodies and the maximum load was observed, which indicates that the increase of bone mass was associated with the increase in bone strength. These findings indicate that activin, as a coupling factor, might be applicable for the treatment of bone fractures and osteoporosis (Sakai & Eto 2001).

Metastasis is defined as a spread and growth of tumour cells to distant organs and is considered to be the most devastating attribute of cancer. It is difficult to find the effective therapy that would be able to eliminate this condition because the knowledge of cellular mechanisms underlying bone metastasis is insufficient (Kang et al. 2003). Breast cancers as well as prostate cancers frequently metastasize to bone, where they disrupt normal bone remodelling, leading to bone disruption, pathological fracture, pain, hypercalcaemia and nerve compression. Cancer cells that metastasize to bone secrete factors such as parathyroid hormone-related protein (PTHrP) and IL-11, which are able to stimulate the osteoclastic bone destruction and activation and release of growth factors immobilized in bone matrix. Released growth factors promote tumour growth and bone disruption (Dunn et al. 2009). TGF-β plays a complex role in malignancy, as it is considered to be a tumour suppressor and inducer in the early and late stage of the pathology, respectively (Fournier & Guise 2007). It plays a crucial role in the feed-forward stimulation of osteoclastic bone resorption, referred to as the vicious cycle of bone metastasis (Fournier & Guise 2007). TGF-β promotes bone metastasis, by regulation of many of the tumour-secreted factors that stimulate tumour growth and bone destruction like PTHrP, IL-11, CXC chemokine receptor 4 (CXCR4), connective tissue growth factor (CTGF) and others. These TGF-β-regulated factors participate in multiple steps of the metastatic cascade, including invasion, doming, angiogenesis and osteolysis. Furthermore they constitute a gene signature for tumours that metastasize preferentially to bone. Unfortunately, standard bisphosphonate therapies improve skeletal morbidity by reduction of osteolysis, but do not cause the regression of established bone metastases (Dunn et al. 2009). For that reason the TGF-β signalling pathway in tumour cells is considered to be a promising therapeutic target, so different modalities to block TGF-β signalling are under investigation. The inhibition of TGF-β signalling by the overexpression of inhibitory Smad 7, or by knockdown of Smad 4, or the treatment with pharmacological inhibitors such as SD-208, an ATP-competitive inhibitor of TGF type I receptor decreased bone metastases in animals models (Dunn et al. 2009). Moreover tissue hypoxia, characteristic for bone microenvironment, activates hypoxia-inducible factor-1α, which also activates many factors, promoting a feed-forward metastatic cycle. For that reason, the combination therapy employing inhibitors of both hypoxia and TGF-β may improve the treatment of patients suffering from bone metastases (Dunn et al. 2009).

It has been found that a number of human cancers are associated with an altered expression level of activin A, or its specific receptors or inhibitors, suggesting its potential role in tumour progression (Leto et al. 2006). Because activin A participates in the regulation of osteoblastic activity as well as osteoclastic differentiation, it has been hypothesized, that it can participate in bone metastasis. Leto et al. (2006) investigated the clinical significance of the serum level of activin A in patients with breast and prostate cancers, which frequently metastasize to bone tissue.

It was found that activin A serum concentrations were increased in patients with breast and prostatic cancer. Furthermore the increased serum level of this cytokine was positively correlated with the number of skeletal metastases, indicating that it may be involved in the pathogenesis of bone metastasis. For that reason this cytokine may be considered as a potential biochemical marker, as well as a potential target for therapeutic approach in the treatment of metastatic bone disease (Leto et al. 2006).

In conclusion, cytokines from the TGF-β superfamily play a significant role in diverse areas of biology.

They control various cellular processes including cell proliferation, cell death, metabolism, homeostasis and differentiation, as well as immune responses and endocrine functions (Harrison et al. 2005). Moreover members of the TGF-β superfamily play an important role in bone tissue biology. Bone homeostasis is controlled by the mutual interaction between osteoblasts and osteoclasts. This interaction is coordinated by hormones as well as by TGF-β superfamily peptides involved in divergent aspects of cellular proliferation and differentiation (Hashimoto et al. 1992). There are many reports presenting the biological role of TGF-β, BMPs, activin A and inhibins in bone tissue. Furthermore several studies present the involvement of TGF-β and activin in osteoporosis and metastatic bone diseases.

TGF-β, activin A, inhibins and BMPs play important roles in bone physiology influencing bone growth, turnover, bone formation and cartilage induction. TGF-β is considered to be a physiological regulator of osteoblast differentiation and a key mediator of the coupling of osteoblast differentiation to osteoclastic bone resorption required for skeletal homeostasis (Filvaroff et al. 1999). There are many studies presenting the involvement of activin A in bone formation; however there are also reports demonstrating an inhibitory activity of activin A on osteoblast differentiation (Eijken et al. 2007). Also, inhibins are important in regulation of bone turnover and bone quality (Perrien et al. 2006, 2007). Both *in vivo* and *in vitro* studies have proved that inhibins exert a bimodal effect on the regulation of bone metabolism (Perrien et al. 2007). Extracellular antagonists of the TGF-β superfamily members are considered to be the important regulators of their signal transduction pathway. These antagonists, including, for example, follistatin, noggin or chordin, are able to bind ligands with high affinity, regulating many physiological responses (Thompson et al. 2005). Follistatin is known to be the pluripotential protein that is able to modulate the activity of members of the TGF-β superfamily, emphasizing the diverse biological properties attributed to that protein. Follistatin, being the antagonist of activin A and BMPs, actively participates in regulation of bone metabolism. It is able to neutralize their activity, emphasizing its important role in bone tissue physiology. However some broader aspects of follistatin physiology are critical for understanding this action. Experiments performed on mice with overexpression of follistatin might be essential for understanding the involvement of follistatin in the regulation of bone metabolism. Furthermore as cytokines from the TGF-β superfamily have profound effects on many tissues, in both physiological and pathological processes, they should be intensively studied. There is a need for more sophisticated understanding of signalling pathways of members of the TGF-β superfamily and interactions of some components and modulators that have relevance for normal physiological processes, as well as in disease states.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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