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## Targeting Fas in osteoresorptive disorders

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## **Abstract**

### **Importance of the field**

Fas receptor is a mediator of the external apoptotic pathway in many cells and tissues. It is proposed to mediate osteoresorptive effects of estrogen deficiency and local/systemic inflammation.

### **Areas covered in this review**

This review covers the past two decades of research on the expression and function of the Fas/Fas ligand system on bone cells, involvement in the pathogenesis of osteoresorption, and potential therapeutic modulation.

### **What the reader will gain**

We review the structure, biological function and intracellular signaling pathways of the Fas/Fas ligand system, emphasizing the role of the non-apoptotic signaling pathways in bone cells, particularly osteoblast differentiation. We also present data on the *in vitro* expression and function of the Fas/Fas ligand system on osteoblast/osteoclast lineage cells, animal and human studies confirming its involvement in osteoresorptive disorders, and potential therapeutic approaches to modulate its function.

### **Take home message**

Tissue specific therapeutic approaches need to be established to modify the Fas/Fas ligand system in osteoresorptive disorders, since systemic targeting has many side effects. The most promising approach would be to target Fas signaling molecules coupled with osteoblast/osteoclast differentiation pathways, but a precise definition of these targets is still needed.

## **1. Introduction**

### **1.1. Structure, expression and function of Fas and Fas ligand**

“Death receptor” is a term for members of the tumor necrosis factor (TNF) family of receptors responsible for the execution of the extrinsic apoptotic pathway and characterized by an intracellular  $\alpha$ -helical protein-protein interaction domain named the death domain (DD). Eight members of the group have been described so far, including a prototypical death receptor Fas (FS-7-associated surface antigen; CD95; APO1; TNF receptor superfamily member 6, TNFRSF6).

Fas is a type I transmembrane protein expressed by numerous healthy and tumor cells and tissues, including B and T lymphocytes, dendritic cells, thymocytes, macrophages, cardiomyocytes, hepatocytes, and various cells within the kidney, pancreas, and brain (1-3). The extracellular portion of the Fas protein comprises three cystein-rich domains characteristic for the TNF superfamily, while the intracellular portion includes the aforementioned death domain (4). A splicing variant of Fas lacks the transmembrane domain and is consequently shed as a soluble receptor, the form that probably has some regulatory functions, competing with membrane-bound Fas for Fas ligand (5).

Fas is specifically activated by its ligand – Fas ligand (CD178; CD95L; APO1L; TNF ligand superfamily member 6, TNFSF6), a type II protein (6, 7). The expression of Fas ligand is more restricted, particularly to lymphocytes T (mainly CD8<sup>+</sup> but also some CD4<sup>+</sup>) and natural killer (NK) cells (8). It is worth mentioning that Fas ligand is also substantially expressed by immunologically privileged tissues such as the eye, testis, and placenta (9).

The primary or the most biologically active form of Fas ligand is a trimer on the cell surface but two other forms have also been documented. The extracellular, TNF-homologous domain

of Fas ligand can be cleaved from the membrane by metalloproteinases, thus resulting in a secreted trimer, which seems to be functionally quite inert and could have a regulatory role by occupying the binding sites on Fas (10). Furthermore, Fas ligand can also be secreted as membrane-bound, in a form of specific microvesicles, which are thought to be biologically active although their exact role is still not quite clear (11). Although the main receptor of Fas ligand is Fas, the decoy receptor 3 (DcR3), another member of the TNF receptor superfamily, is also able to bind Fas ligand, thus functioning as a regulator of the Fas–Fas ligand pathway (12). Interestingly, there are reports showing that Fas ligand per se can act as a receptor by reverse signaling through its intracellular tail (13).

The first step of the Fas intracellular signaling pathway is the trimerization of Fas within the lipid rafts (14). Trimers of Fas–Fas ligand are subsequently oligomerized, which brings the intracellular parts of the Fas receptor in close proximity. This clustering triggers downstream signaling (Fig. 1): the DD of Fas binds a small adapter molecule, Fas-associated death domain (FADD)(15). FADD is characterized by the death effector domain (DED) which, through homotypic interactions, recruits pro-caspase-8 and pro-caspase-10, forming a death-inducing signaling complex (DISC), the molecular platform for initiation of Fas-mediated events (16). The formation of DISC results in autoproteolytic activation of caspase-8, and formation of an active caspase-8 heterotetramer which is released into the cytosol to activate the effector caspase-3 (17). Besides aforementioned, there are additional molecules which have been described to recruit to DISC (i.e. DAXX, FAP-1, FLASH, RIP, FAF-1), but their precise roles still need to be elucidated (18). DISC formation is an important regulatory instance of the Fas pathway where processing of procaspase-8 can be blocked by c-FLIP (cellular FLICE [=caspase-8, FADD-like interleukin-1 $\beta$ -converting enzyme] inhibitory protein), which interacts with FADD through DED (18). FADD can also bind to a transmembrane protein TOSO, another anti-apoptotic factor (19).

Subsequent intracellular events are the basis for the classification of cells in two types with respect to the involvement of the mitochondrial (internal) pro-apoptotic machinery (18). (Fig. 1) Type I cells (such as lymphocytes) are characterized by abundant DISC formation, rapid activation of executor caspases and subsequent cell death. Type II cells (e.g. hepatocytes) are not as sensitive to Fas ligand, possibly because of an early exclusion of receptors from the lipid rafts (20). The amounts of activated caspase-8 are relatively low, so the apoptotic signal needs to be amplified through the internal (mitochondrial) apoptotic pathway (17).

The ability of Fas to mediate cell death is best observed in the immune system, where it contributes to the shutdown of chronic immune responses and to the maintenance of peripheral tolerance, by a process known as restimulation-induced cell death (RICD) (21). Within the course of a prolonged immune response the availability of growth factors as well as diminished stimulation by antigen receptors make T and B cells susceptible to various apoptotic stimuli (16). Moreover, repeatedly stimulated cells upregulate their expression of Fas ligand (22) thus killing themselves (suicide) and neighboring T cells (fratricide) as well as B cells, dendritic cells, and macrophages (23, 24). In addition to the lymphocyte homeostasis, Fas–Fas ligand is an executive mechanism used by the effector arm of the immune response to combat infections. Fas ligand is expressed by both CD8<sup>+</sup> T cells and NK cells (25) and it contributes mostly to the killing of virus-infected cells (26) and possibly some tumor cells (27). It is also worth to mention that CD4<sup>+</sup> cells could suppress the CD8<sup>+</sup> cells by the Fas pathway (28).

Although apoptosis is the most striking outcome of Fas – Fas ligand interaction, their biological effects not based on cell death should be emphasized. Fas/Fas ligand interaction increases the production of chemokines in NK-T cells (29), increases also the activation of dendritic cells, and increases cellular proliferation (30). Fas ligand is a pro-survival factor for human CD34<sup>+</sup> cells (31) and augments their colony-forming capacity (32).

Having in mind the various roles of the Fas–Fas ligand system, it is not surprising that its effects are not mediated only by the cells' apoptotic machinery but via other signaling pathways as well. The intracellular portion of Fas receptor contains a tyrosine-containing motif tyrosine–x–x–leucine, similar to the canonical immunoreceptor-tyrosine-activation-motifs (14). Upon ligand binding a phosphatidylinositol-3-kinase (PI3)-activation complex (PAC) is formed, with subsequent phosphorylation of a number of intracellular targets (33). Although the functional consequences are still poorly understood, this pathway is caspase-8-independent, and does not lead to apoptosis but, at least in the model of a glioblastoma cell line, promotes cellular migration (14). Increased motility and invasiveness of tumor cell lines have also been described as a result of Fas-mediated activation of nuclear factor (NF)  $\kappa$ B pathway (34). Depending on the subtle balance between procaspase-8 and c-FLIP at the DISC, engagement of Fas could lead to either caspase-mediated cell death, or triggering of NF $\kappa$ B signaling and cellular activation (35). Caspases are also confirmed to cleave the components of the NF $\kappa$ B pathway, and thus, regulate cellular proliferation responses (36). Finally, several groups reported on involvement of another major group of signaling molecules, i.e. mitogen activated protein kinases (MAPK) (37, 38) in Fas-mediated systems.

## **2. Involvement of Fas in osteoresorption**

### **2.1. Expression and function of Fas on the cells of the skeletal system**

Bone remodeling is a continuous process in which bone is resorbed by osteoclasts and formed by osteoblasts, to maintain bone mass. Osteoblasts develop from mesenchymal progenitors present in bone marrow as well as in various tissues such as trabecular bone, fat, synovium, skin and umbilical cord blood (39). Osteoclasts develop from multipotent monocyte/macrophage hematopoietic progenitors whose differentiation towards the osteoclast



lineage is determined by binding of receptor activator of NF- $\kappa$ B ligand (RANKL) to the receptor activator of NF- $\kappa$ B (RANK) expressed on their surface (40).

Many studies investigated the expression and function of Fas on cells involved in bone homeostasis. Fas is expressed on both murine and human osteoblasts (41, 42) and osteoclasts (43), but the data on the expression levels at certain osteoblast or osteoclast maturation stages, and function of Fas are conflicting.

Several groups reported on the expression of Fas on mesenchymal stem cells as osteoblast progenitors. For instance, Fas protein expression has been confirmed by flow cytometry on human umbilical cord-derived mesenchymal stem cells (44), and several murine bone marrow-derived stromal cell lines, where agonistic antibodies were able to induce apoptosis (42). Strong expression of Fas gene was also detected in primary murine bone marrow-derived stromal cells (45).

Moreover, the expression of Fas on immature and mature osteoblasts was confirmed by a number of research reports. Strong constitutive expression of Fas has been demonstrated on the cells belonging to a human osteosarcoma cell line MG63, and primary human osteoblasts, where IgM anti-Fas antibody was able to induce apoptosis (46). In another report, MG63 cells and mature human osteoblasts differentiated from human mesenchymal cells weakly expressed Fas mRNA, as detected by gene-array hybridization (41). Murine preosteoblast MC3T3 cells did not express Fas (47). Expression of Fas gene seems to appear in mature osteoblasts, and increases in the calcification phase (48). On the protein level, as detected by flow cytometry, Fas appears on approximately one third of primary murine osteoblasts as they begin to differentiate and this percentage increases with osteoblast maturation (48).

Data on the function of Fas expressed on osteoblast lineage cells are variable, which can be partially explained by the fact that the expression and function of Fas on the osteoblast lineage

cells may be affected by various cytokines and growth factors, which modify osteoblast sensitivity to Fas-induced apoptosis. Differences in expression and function of Fas among various studies can be ascribed to the variability in composition of animal sera and media used for cell culture among laboratories. For instance, IGF I added *in vitro* stimulates proliferation of human osteoblasts, but it also increases expression of Fas and induces apoptosis of proliferating osteoblasts (49). Although mouse preosteoblast cell line MC3T3 does not express Fas in basal conditions, addition of TNF- $\alpha$ , interleukin (IL)-1 $\beta$  and interferon (IFN)- $\gamma$  sensitize it to apoptosis induced by the addition of an agonistic anti-Fas antibody (47). Intracellular mechanisms involved in the regulation of the external apoptotic pathway may also modify the sensitivity of osteoblast lineage cells to Fas-induced apoptosis. E.g., vitamin 1,25(OH) $_2$ D $_3$  is able to block anti-Fas antibody-induced primary human calvarial osteoblast apoptosis, interfering with the intracellular apoptotic signaling (50). MG63 cells express Fas, but presence of IFN- $\gamma$  is required for Fas-induced apoptosis (51). According to our results, Fas activation induces apoptosis in both immature and mature bone marrow-derived murine osteoblastogenic cultures, but not in all Fas-expressing cells. However, the addition of Fas ligand to osteoblastogenic cultures reduces the number of osteoblast colonies, which could not be ascribed to apoptosis, since the number of total colonies was not reduced. Partial resistance to apoptosis and the specific reduction in the number of osteoblast colonies suggests a non-apoptotic role of Fas in regulation of osteoblast differentiation and maturation (48).

The data on Fas expression and function on osteoclast lineage cells are even more conflicting. Wu et al. found strong expression of Fas gene and protein in primary murine, human, and avian osteoclasts that increased with osteoclast differentiation (43). Moreover, human cord blood monocyte-derived osteoclasts have been shown to express Fas, and their apoptosis could be induced by the addition of anti-Fas agonist antibody (52). In other reports, Fas was

undetectable by flow cytometry in human peripheral blood-derived osteoclasts (53).

According to our data, the transcriptional activity of Fas gene was almost absent in murine bone marrow-derived primary osteoclasts, and Fas protein was found to be weakly expressed on approximately 10% of these cells (48). Similar results were obtained by Park et al. who described a weak expression of both Fas and Fas ligand on primary murine bone marrow-derived osteoclasts (54). Functional studies also provided contradictory results: increased osteoclast apoptosis is reported by Wu et al. upon addition of Fas-activating antibody to osteoclast cultures (43), whereas enhanced osteoclastogenesis was reported by Park et al. upon addition of recombinant Fas ligand, which was explained as a response to increased production of IL-1 $\beta$  and TNF- $\alpha$  by surrounding cells (54). There are additional reports showing a constitutive expression of Fas on murine bone marrow-derived osteoclasts, whose apoptosis may be induced by the up-regulation of Fas ligand by estrogen in the same cells (55). Expression and function of Fas is proposed to be biphasically regulated by RANKL (56). RANKL upregulates the expression of Fas in osteoclast progenitors, and may negatively regulate the size of the osteoprogenitor pool, whereas in mature osteoclasts, RANKL decreases expression Fas and prolongs their survival (56).

The source of Fas ligand which can affect bone cells is still under debate but likely candidates are activated T and B lymphocytes, monocytes/macrophages, and NK cells (8). In addition, our group detected very low levels of Fas ligand mRNA and protein in murine osteoblasts and osteoclasts, i.e. within the cells of the bone milieu (48). However, according to Nakamura et al., Fas ligand is expressed by mature osteoclasts, and may be upregulated by estrogen which thus decreases their life span and prevents bone loss (55). On the other hand, Krum et al. documented, *in vitro* and *in vivo*, an estrogen-inducible expression of Fas ligand on osteoblast lineage cells. They also showed that osteoblasts are required for human pre-osteoclast apoptosis induced by estradiol suggesting that paracrine effects of FasL-expressing

osteoblasts are required for bone protective effects of estrogen (57). Several cytokines can modulate Fas expression and activation in osteoclasts, e.g. TNF stimulates osteoclastogenesis and increases osteoclastic expression of Fas in vitro (58). Conversely, presence of IL-12 or IL-18 may abolish increases in osteoclastogenesis induced by TNF, by upregulating Fas ligand in cultured cells (58, 59).

## **2.2. Mouse models for studying the role of the Fas/Fas ligand system in osteoresorptive disorders**

Biological function of Fas and Fas ligand as regulatory molecules in the immune system has been confirmed and extensively studied using mouse models with spontaneous or induced mutations.

There are two spontaneous mutation models for studying the Fas/Fas ligand system, and several induced mutations, i.e. knock-out models for either or both of the molecules. A spontaneous mutation in the coding for Fas ligand gene on chromosome 1 [a T-to-C transition point mutation that causes a replacement of a highly conserved phenylalanine with a leucine at position 273 in the extracellular region of the encoded protein (60)] has first been described in the C3H/HeJ strain (61). Mice homozygous for this mutation are unable to express a fully functional Fas ligand and develop lymphadenopathy and systemic autoimmune disorder, typically with enlargement of all lymph nodes, spleen, and increased numbers of T and B lymphocytes and dysregulation of their maturation and function (62). Due to their phenotype – generalized lymphoproliferative disorder – the mutant mice were named *gld*. A spontaneous mutation in the coding region of the Fas gene on chromosome 19 (63), first described in the MRL strain of mice (64), presents as a disorder very similar to the *gld* phenotype called a lymphoproliferative syndrome or *lpr*. One of the characteristics of both models is the

progressive accumulation of non-malignant CD4<sup>-</sup>CD8<sup>-</sup> T lymphocytes. Because of the similarities of the two phenotypes it has early been postulated that these genes represented a receptor-ligand pair (65).

Although both of these point mutations lead to an abolishment of Fas ligand and Fas function, respectively, it has been shown that both mutations may be leaky. The leakiness of the *lpr* mutation was shown (66) because small amounts of intact mRNA for Fas were found in immunocompetent tissues of *lpr* mice (e.g. thymus), and the leakiness of the *gld* mutation was long suspected because the *gld* phenotype did not correspond well to that of the Fas knock-out mutant mice (67). Hence, the introduction of knock-out strains (Fas-null mice by Adachi et al, and Fas ligand-null mice by Karray et al) helped to fully characterize the role of the system and the phenotype (68, 69). The most striking differences in phenotypes of induced models with a complete lack in Fas/Fas ligand signaling compared to mutant models are in the level of lymphoproliferation and the involvement of the liver; although the phenotypes are strain-specific (e.g. the MRL strain shows the most severe phenotype).

As the largest part of the cells of the skeletal system undergo apoptosis – up to 80% of osteoblasts (51), and most of osteoclasts (70), it has long been postulated that the Fas/Fas ligand system is involved in the regulation of, at least, some aspects of bone cell proliferation and cell death. As results of *in vitro* experiments on the expression and function of either molecule in cells of the skeletal system are often contradictory, mouse models lacking components of Fas/Fas ligand system have been used to study their regulatory effects on bone homeostasis. Since osteoresorptive disorders have a complex pathogenesis, involving several organ systems and multiple cellular interactions on various levels, studies on animal models of osteoresorption are necessary to confirm the physiological importance of a single molecule or molecular system in the pathogenic process. There are two widely used models for systemic and localized osteoresorption: 1) estrogen withdrawal by ovariectomy (ovx), and 2)

murine arthritic disease accompanied by localized osteodestruction and systemic osteoresorption. Estrogens are important regulators of both the immune system (71) as well as bone (72), and modification of levels of estrogen is an invaluable model for the analysis of the Fas/Fas ligand system (73). It has been shown *in vivo* that the dysregulation in the Fas/Fas ligand system protects bones from the effects of estrogen withdrawal (55, 74, 75), i.e. bone loss. Joint destruction in arthritic diseases is a consequence of synovial hyperplasia which leads to formation of tissue-invasive pannus, increased osteoclastogenesis followed by localized bone resorption, and destruction of articular cartilage by inflammatory cells (76). Furthermore, hyperplastic synovia contains mesenchymal cells with a reduced ability to differentiate into mature mesenchymal lineage-cells (osteoblasts, chondroblasts, and adipocytes) (77). Impaired mesenchymal differentiation may be responsible for decreased osteoblastogenesis and may also contribute to subchondral bone loss in arthritis. However, studied on *in vivo* models, the role of Fas/Fas ligand system in the arthritic joint destruction is controversial, and points to the influence of genetic factors on the pathogenesis of arthritis. For example, the MRL-*lpr* mouse strain constitutively develops autoimmune arthritis in approximately 20% of animals (78). On the other hand, it has been shown that collagen induced arthritis is less severe in C57BL/6 mice with an *lpr* mutation and that this finding cannot be explained by the suppression of immune responses (79). Such differences can be also ascribed to the differences in cellular populations affected by Fas deficiency between different genetic backgrounds.

### **2.3. The role Fas ligand/Fas system in human diseases**

Although the Fas/Fas ligand system has been recognized as one of the key pathways in controlling the proliferation and cell death in many sites and tissues, human diseases

involving mutations of components of this system are quite rare. These spontaneous mutations are described as an autoimmune lymphoproliferative syndrome (ALPS), and could be subdivided into four classes, with respect to the affected molecule (80). The syndrome is characterized by lymphadenopathy and splenomegaly, which are most often accompanied by autoimmune hemolytic anemia, thrombocytopenia and other autoimmune manifestations (81). In addition, the Fas/Fas ligand system has also been shown to contribute to several human autoimmune diseases such as autoimmune thyroiditis, systemic lupus erythematosus, and rheumatoid arthritis (82). Moreover, different studies point to the role of Fas/Fas ligand in the regulation of bone metabolism, implicating the role of Fas ligand/Fas malfunction in the pathogenesis of postmenopausal osteoporosis and osteoresorption in rheumatoid arthritis. Developing strategies to modulate these molecules for therapeutic purposes have become a promising challenge (83-85).

Postmenopausal osteoporosis is the most common form of osteoporosis in which estrogen deficiency gives rise to an imbalanced high bone turnover, characterized by an enhanced osteoclast activity and a temporal increase in osteoblast activity which is not able to balance osteoclast-mediated bone resorption (86). Although various systemic and local regulators are involved in osteoporosis caused by estrogen deficiency, including activated T lymphocyte-associated osteoclast stimulation by RANKL and TNF- $\alpha$  (87), the Fas/Fas ligand pathway emerged as an intriguing multifunctional regulatory system. In addition to data from animal models that implicated the role of Fas activation in osteoporosis following ovariectomy by several mechanisms (55, 57, 74, 75), some reports emphasized the importance of the Fas/Fas ligand system in the pathogenesis of human postmenopausal osteoporosis (88, 89). It has been shown that human postmenopausal osteoblasts constitutively express Fas receptors on cell surface, whose activation is able to induce their apoptosis (88). The same study also demonstrated that lethal effects of Fas ligand are exacerbated in the presence of TNF.

However, therapeutic concentrations of estradiol or of estrogen receptor modulator raloxifene analog did not affect the sensitivity of postmenopausal osteoblasts to Fas-mediated apoptosis. Nakamura et al (55) showed that estrogen induces apoptosis and upregulates Fas ligand expression in murine osteoclasts. As mentioned before the findings regarding the functional activity of Fas in human osteoclast differentiation and survival are still controversial (43, 52, 53). Nevertheless, it is possible that estrogen regulates the life span of human osteoclasts via the induction of the Fas/Fas ligand system, offering the possibility that the induction of Fas would decrease bone resorption. In these circumstances, the likely sources of Fas ligand are immune cells, particularly activated T lymphocytes. Yamaza et al recently demonstrated osteoporosis mechanism in which activated T lymphocytes, via Fas pathway, induced both mouse and human bone-marrow mesenchymal stem cell apoptosis (89).

Emerging evidence suggests that impaired Fas/Fas ligand system plays a role in the pathogenesis of rheumatoid arthritis (RA). The main feature of RA is synovial hyperplasia, which leads to cartilage and bone damage in the inflamed joints. Synoviocytes, synovial T lymphocytes and macrophages from arthritic joints express high levels of Fas and/or Fas ligand, and *in vitro* are highly susceptible to Fas/Fas ligand induced apoptosis (90). In contrast, resident synoviocytes are resistant to Fas-related apoptosis, probably reflecting the presence of multiple anti-apoptotic factors in the rheumatoid synovium, such as soluble Fas ligand and Fas, anti-apoptotic cytokines [transforming growth factor (TGF)- $\beta$ , basic fibroblast growth factor (bFGF)] or Fas pathway inhibitors (matrix metalloproteinase (MMP)-2, FLIP) (90). Moreover, immune cells within inflamed joints are resistant to Fas ligand-mediated apoptosis, which leads to their accumulation and creates sustained inflammation that promotes osteoresorption (91). Reduced susceptibility to Fas-mediated apoptosis may also contribute to the expansion of an activated CD4<sup>+</sup> T lymphocyte subpopulation and thus to the maintenance of peripheral autoreactive T lymphocyte clones in RA (92). In addition, our



group demonstrated decreased expression of Fas ligand in the peripheral-blood mononuclear cells of patients with RA, as well as a negative correlation of Fas ligand expression with the clinical assessment of disease activity (93). Furthermore, we also showed the non-apoptotic role of Fas in inhibition of osteoblast differentiation (48). Since proliferating, pannus-forming mesenchymal cells are resistant to apoptosis, Fas may, in these cells, have additional negative effects on subchondral bone loss, inhibiting osteoblastic bone formation. On the other hand, some authors suggested that susceptibility of RA osteoblasts to cytotoxic action of Fas ligand, produced by activated peripheral-blood mononuclear cells, underlies osteoporosis in RA (94). Similar to the animal models, the human studies also point to the still controversial role of the Fas/Fas ligand pathway in human disorders accompanied by osteoresorption, and caution is warranted in the use of Fas as a therapeutic target, as some patients with inflammatory arthritides may benefit from blockade, while others may benefit from agonism of Fas (95).

#### **2.4. Therapeutic approaches to modulate Fas/Fas ligand pathway in osteoresorptive disorders**

Osteoresorptive disorders develop when bone resorption by osteoclasts overcomes the bone formation by osteoblasts. Since Fas is shown to be expressed under various conditions on osteoblasts and osteoclasts, therapeutic approaches, both agonizing and antagonizing Fas signaling may be helpful in the treatment of osteoresorptive disorders, i.e. promoting Fas activation and apoptosis in osteoclasts, and inhibiting Fas mediated apoptosis in osteoblasts.

Therapeutic approaches targeting the Fas/Fas ligand system which include systemic treatment with antibodies to Fas or with Fas ligand, revealed not to be a feasible strategy owing to the nonselective activation of Fas receptor on both, osteoblast and osteoclast lineage cells, as well as due to the hepato- and severe systemic-toxicity, pulmonary inflammation and fibrosis (84,

96, 97). There are relatively non-toxic modulators of the Fas death pathway which can be applied systemically, such as nonsteroidal anti-inflammatory drugs, methotrexate and anti-TNF-directed therapies (98-101), and might prove interesting in the context of the role of Fas ligand/Fas system in bone homeostasis. A pharmacologic stem cell-based intervention by aspirin is also suggested as an approach for osteoporosis treatment, since T lymphocyte activation and Fas-induced mesenchymal stem cell apoptosis could be blocked by aspirin *in vitro* (89). Epidemiological studies reported that regular use of aspirin or non-steroidal anti-inflammatory drugs may have a moderate beneficial effect on bone mineral density in postmenopausal women (98). However aspirin may contribute to multiple biological pathways, which makes it difficult to elucidate its precise functional mechanism in relation to bone remodeling.

Direct targeting of osteoclast and osteoblast differentiation and influencing their life span to treat osteoresorptive disorders, including the modulation of the Fas ligand/Fas system, may be a more specific and potent therapeutic approach.

As shown by studies related to Fas pathway modulation in cancer, Fas ligand gene may be directly delivered to target cells, with minimum systemic leakage (102). More recently, interfering RNA technology has been utilized to target gene expression of members of the extrinsic death receptor/Fas pathway in septic conditions, and the same approach may be used to target these members in osteoblast/osteoclast lineage cells. The experience with double stranded small interfering RNA (siRNA) against Fas and caspase-8 is an intriguing approach, but several limitations beyond cell targeting need to be resolved before siRNA can be applied clinically. Obstacles may include the induction of IFN signaling, since siRNA is a double stranded RNA, and subsequent unwanted inflammatory reactions (103).

As suggested by specific modulation of signaling pathways in tumors of tissue cells (18, 85, 104, 105), tissue specific interventions directly aiming the Fas downstream signaling pathway, which is at the same time critical for osteoblast/osteoclast differentiation such as Raf/MEK/ERK, NF- $\kappa$ B and JNK/Jun, Wnt, and Smads, should be appreciated as more powerful and promising approaches (Fig. 2).

Among the first were agonistic therapeutic interventions on Fas in arthritis, where hyperplastic synoviocytes destroy the subchondral bone. Since synovial inflammation is characterized by the unique resistance of proliferating synoviocytes to Fas-related apoptosis, Fas activation in these cells has drawn much attention as a possible tool to prevent joint destruction in both animal and human inflammatory arthritides (90). In line with this idea is the study that investigated apoptosis resistance caused by defects in the extrinsic and intrinsic apoptotic pathways in RA (106). Inhibition of PI3 kinase sensitizes RA fibroblast-like synovial cells to Fas-induced apoptosis by increasing cleavage of Bid protein, whose suppression is able to completely abrogate Fas-induced apoptosis. Bid overexpression increased the sensitivity of fibroblast-like synovial cells to Fas induced apoptosis, in association with cleavage of caspase-9. In RA fibroblast-like synovial cells, phosphorylation of Akt protects against Fas-induced apoptosis through inhibition of Bid cleavage. The study stressed that the connection between the extrinsic and the intrinsic apoptotic pathways is critical in Fas-mediated apoptosis and pointed to PI3 kinase as a potential therapeutic target for RA. Based on our recent findings in a mouse model of Fas deficiency, Fas activation seems to suppress osteoblast differentiation and contributes to osteoresorption in osteoporosis and subchondral bone loss in RA (48, 75). Inhibition of osteoblast differentiation was dependent on caspase-8 activation and resulted in down-regulation of expression of Runx2 gene, one of the major transcription factors involved in osteoblast differentiation (107), suggesting that activated caspase-8 may have a specific regulatory function in osteoblast

lineage cells, and inhibition of caspase-8 may present an approach to prevent osteoresorption. A number of small molecule caspase inhibitors, such as IDN-6556, a pan-caspase inhibitor, are already in phase II clinical trials for treatment of liver disease (108). Regulatory function of caspases in osteoblast differentiation has already been confirmed for caspase-3, whose deficiency resulted in impaired osteoblastic differentiation of bone marrow stromal cells due to an overactivation of the TGF- $\beta$ /Smad2 signaling pathway (109). Caspases may also inhibit the NF- $\kappa$ B survival pathway via the cleavage of the I $\kappa$ B kinase (IKK) complex scaffold protein and NF- $\kappa$ B modulator NEMO (36). The cleavage of intracellular proteins involved in differentiation pathways, may result in inadequate differentiation signals in osteoblast lineage cells, induced by Fas. Definition of caspase targets within these pathways may provide a specific pharmacological approach to counter-regulate osteoresorption.

Having in mind that both Fas ligand and RANKL belong to the TNF superfamily of molecules, it is not surprising that in osteoclast lineage cells their intracellular signaling greatly overlaps (16, 110). It has been shown that their intracellular pathways interact with each other resulting in the modulation of Fas signaling by RANK engagement. RANKL provides osteoclast survival signals through several pathways, including c-src, PI3-kinase/Akt, and the caspase cascade (56). RANKL also upregulates NF- $\kappa$ B activity in osteoclast precursors, as well as binding of NF- $\kappa$ B to the Fas promoter. RANK also signals by increasing Ca<sup>2+</sup> influx and activation of the calmodulin-dependent phosphatase, calcineurin, which dephosphorylates nuclear factor of activated T-cells (NFAT), leading to NFAT translocation to the nucleus and activation of downstream osteoclast gene expression (Fig. 2). At the same time calmodulin regulates Fas-mediated apoptosis, at least in part, by binding to the death-inducing signaling complex inhibitor FLIP (111). Novel anti-resorptive drugs can target both Fas-mediated and RANKL-mediated pathways. For example, Ikarisoside A, isolated from *Epimedium koreanum* (Berberidaceae), has inhibitory effects on the RANKL-

mediated activation of NF- $\kappa$ B, JNK, and Akt, decreasing the expression of c-Fos and NFATc1 as well as osteoclast-specific genes like matrix metalloproteinase 9 (MMP9), tartrate-resistant acid phosphatase (TRAP), RANK, and cathepsin K (112).

## **2.5. Conclusions**

Fas is a member of the death receptor subgroup of the TNF receptor superfamily, activated upon binding of its ligand – Fas ligand that belongs to the TNF superfamily of ligands. The significance of the Fas/Fas ligand system has been extensively studied and recognized as important for the limitation of the chronic immune activation, maintenance of peripheral tolerance, and effector immune response. Since it is expressed by a variety of cells and tissues, including osteoblasts and osteoclasts at various differentiation stages, Fas may be involved in the regulation of their survival. Apoptosis has been considered as the main consequence of Fas activation, but there is increasing evidence that Fas activation may have additional regulatory, non-apoptotic functions in various cells and tissues.

Although there are still many gaps in our knowledge and understanding of expression and function of Fas in cells of the skeletal system, animal models provide the evidence of Fas involvement in the regulation of bone homeostasis, and confirm the hypotheses that Fas inhibition may have osteoprotective effects in postmenopausal osteoporosis, as well as in rheumatoid arthritis. Studies performed on human subjects with osteoporosis and rheumatoid arthritis also support the involvement of Fas in the pathogenesis of these diseases.

Therapeutic inhibition, or in some cases, activation of Fas receptor may be a promising approach in these conditions. However systemic anti-Fas therapy has many complications and side effects, and therefore, new therapeutic approaches have to be established to modify the Fas/Fas ligand system in human diseases. These approaches include the use of systemic modulators which also affect expression and function of Fas, gene therapy or iRNA.

However, a more specific therapeutic approach would target Fas intracellular signaling molecules coupled with pathways involved in osteoblast or osteoclast differentiation and activity, and their identification is required to fulfill this goal.

### **3. Expert opinion**

Fas receptor is ubiquitously expressed in human and animal tissues, including skeletal tissue cells. However, the precise data on the expression and function of Fas on osteoblast and osteoclast lineage cells are still missing, mostly due to the fact that various reports provide different results and conflicting data, even in the same cell types and differentiation stages of bone cells.

On the other hand, data obtained by animal models confirm that the blockage of Fas has a protective effect in osteoporosis, as well as in inflammation induced joint destruction and subchondral bone formation in RA. The precise mechanism underlying this protective effect is controversial and incompletely defined, since several research groups studying the effects of the Fas/Fas ligand system in bone metabolism provide various explanations based on their data.

Osteoresorption is a consequence of either a decreased bone formation by osteoblasts or an increased bone resorption by osteoclasts. Among osteoresorptive disorders, osteoporosis and rheumatoid arthritis represent major public health problems due to their high morbidity and disability rates. Due to the complexity of both conditions, various therapeutic approaches have been used, which are either partially successful, or burdened with side effects and complications. Development of new therapeutic approaches to these conditions is an important area of research, where modification of Fas receptor-mediated effects may benefit the patients.

Cellular effects of Fas ligation vary, depending on several factors which may be therapeutically modified, including: a) surface expression and function of Fas, which can be altered by various intra- and extra-cellular stimuli, b) cell sensitivity to apoptosis, depending on the presence and activity of various anti-apoptotic molecules, and c) regulatory intracellular networks present and active in a specific cell, responsible for non-apoptotic responses in target cells.

Direct therapeutic targeting of Fas is extremely difficult, due to its broad tissue expression, producing systemic effects. One of the specific approaches to target Fas in osteoresorptive disorders may involve cell therapy including *ex vivo* manipulation of Fas expression, or the quantity and activity of anti-apoptotic and regulatory molecules in osteoblasts, osteoclasts or their progenitors, which would aim to increase osteoclast apoptosis and/or decrease osteoblast apoptosis. However, these therapeutic approaches are also accompanied by various side effects and potentially dangerous consequences related to tumorigenicity of modified cells.

Another approach is to define intracellular targets in skeletal cells and specific conditions (cell type, differentiation, activation stage) in which these targets are critical for the pathogenesis of osteoresorption, thus providing the possibility of pharmacological modulation of their responses to Fas activation. Modulation of apoptotic signaling has been extensively studied in various tumor cells where molecular interfering with survival pathways, such as JAK/Stat, or C-Raf-1, MEK-1 and Erk1/2 kinases, modifying Bcl-2 levels or the PI3K Akt activating pathway, may increase tumor cell sensitivity to conventional therapy. Additionally, caspases with their important role in cell apoptotic machinery, as well as due to their increasingly recognized signaling functions are also potential targets for pharmacological modulation of intracellular responses to Fas activation. Various synthetic or natural molecules with activating or inhibitory effects to the above mentioned pathways have been tested in pre-clinical trials, and minor proportion of them such as Ikarisoside A or IDN-6556 has entered

Phase II clinical trials. This approach may also be beneficial in osteoresorptive disorders, through modifying the life span, differentiation, and activity of osteoblasts and osteoclasts. These alternative molecular components connecting Fas signaling and osteoblast/osteoclast differentiation and activity are still undefined and their functional importance still requires stronger evidence from preclinical studies. A better understanding of these pathways will clarify additional molecular mechanisms leading to osteoresorption, and contribute to the definition of new therapeutic targets.



## Article highlights

- Fas is a receptor from the TNF receptor superfamily, whose activation by binding of Fas ligand leads to apoptosis via external apoptotic pathway. Additionally, non-apoptotic effects of Fas signaling include regulation of cell proliferation, differentiation, and activation.
- Expression and function of Fas on bone forming osteoblasts and bone resorbing osteoclasts has been extensively studied *in vitro*, but various reports often provide conflicting data. Fas and Fas ligand are shown to be expressed at certain stages of osteoblast and osteoclast differentiation, and their expression may be modified by various cytokines. Therefore, the Fas/Fas ligand system may be directly involved in the regulation of bone homeostasis.
- Importance of Fas in osteoresorptive disorders has been confirmed in mice with loss-of-function mutation in Fas, by two animal models of osteoresorption: estrogen withdrawal-induced bone loss and collagen induced arthritis, a mouse model of human rheumatoid arthritis. These findings are in accordance with the results of several studies on postmenopausal osteoporosis and rheumatoid arthritis involving human subjects.
- Since bone loss is a result of shifting the balance between bone formation by osteoblasts and bone resorption by osteoclasts towards resorption, therapeutic approaches promoting Fas activation and apoptosis in osteoclasts, and inhibiting Fas mediated apoptosis in osteoblasts, may be helpful in the treatment of osteoresorptive disorders. Systemic Fas targeting leads to nonselective activation of Fas receptor on both cell types and results in systemic toxic complications. A targeted approach may involve *ex vivo* manipulation of Fas expression in osteoblasts, osteoclasts or their progenitors; or definition of specific intracellular targets of Fas, involved in the regulation of life span, differentiation, and

activity of osteoblasts and osteoclasts, to pharmacologically treat osteoresorption.

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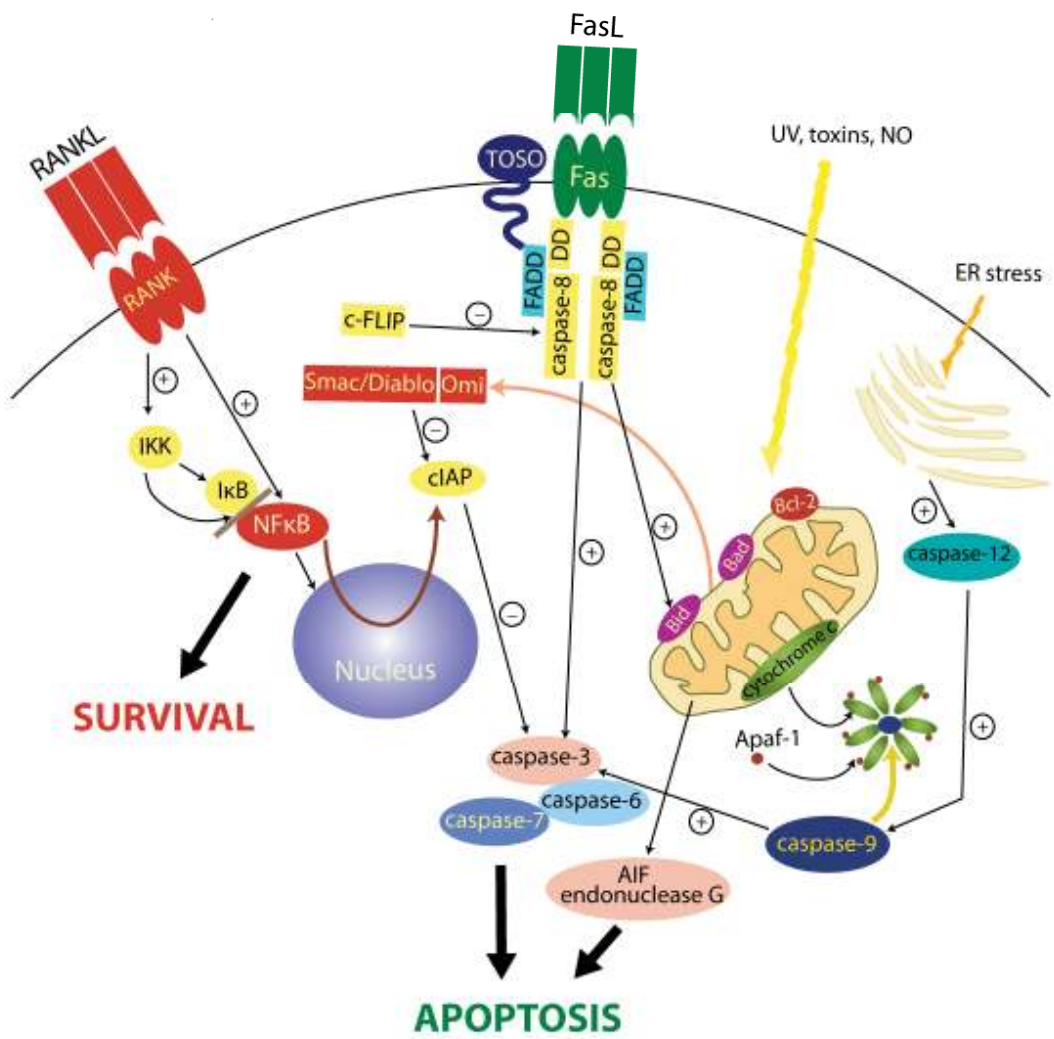
**Figure 1. Schematic presentation of apoptotic pathways and their connection to cell proliferation and survival pathways.**

Fas receptor ligation initiates extrinsic apoptotic pathway which through its death domains (DD) binds small adapter molecule Fas-associated death domain (FADD) thus activating caspase-8 (or FLICE, FADD-like interleukin-1 $\beta$ -converting enzyme). The activated caspase-8 further activates caspase-3 and other effector caspases which lead the cell into apoptosis.

Apoptosis can at this level be prevented by c-FLIP (cellular FLICE inhibitory protein), attaching to DD.

NF $\kappa$ B transcription factors promote cell growth and differentiation. They are activated usually through phosphorylation of inhibitory proteins I $\kappa$ B by I $\kappa$ B kinases (IKK) upon ligand binding of various receptors (i.e. RANKL on osteoclast lineage cells). In the nucleus, NF $\kappa$ B initiates transcription of inhibitors of apoptosis proteins (IAPs), which inhibit the effector caspase-3. On the other hand, binding of IAPs may be prevented by Smac/DIABLO or serine protease Omi, released from a mitochondria by an apoptotic stimulus.

Mitochondria are involved in the intrinsic apoptotic pathway, initiated by cellular stress and leading to the release of cytochrome c from their internal membrane. Together with Apaf-1 (apoptotic protease activating factor-1), cytochrome c forms an heptameric, caspase-9 activating structure - apoptosome. Cytochrome c release is stimulated by the pro-apoptotic members of the Bcl-2 protein family from the Bax subfamily (i.e. Bid and Bad) which destabilize the outer mitochondrial membrane. Anti-apoptotic members (Bcl-2) prevent the activation and effects of the pro-apoptotic Bax proteins. Bax subfamily members may also activate caspases. On the other hand, caspase-8, cleaves and activates the Bid protein, thus transferring the signal from the extrinsic to the intrinsic apoptotic pathway (especially important in type II cells).



**SURVIVAL**

**APOPTOSIS**

**Figure 2. Schematic presentation of osteoblast and osteoclast differentiation pathways and their connection with Fas downstream signaling pathways.**

Osteoblast differentiation is induced by several receptors, and leads to the activation of osteoblast transcription factors, including runt-related transcription factor 2 (Runx2), osterix, and Dlx5. Transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily members (including bone morphogenetic proteins, BMPs) signal via serine/threonine receptor kinases and phosphorylation of Smad molecules. Phosphorylated Smads bind with the common Smad4 and forming a trimeric structure which is translocated to the nucleus, where they induce transcription of osteoblast genes. TGF $\beta$  superfamily receptors also signal through mitogen activated protein kinase (MAPK) pathways. Fibroblast growth factor (FGF) receptor signals through cytoplasmatic parts possessing tyrosine kinase domains, activated upon conformational changes induced by ligand binding. They phosphorylate and activate MAPK and protein kinase C (PKC).

Wnt ligand binds to a frizzled (FZZ)/low density lipoprotein receptor related protein (LRP) complex, activating the intracellular protein dishevelled (DSH). DSH inhibits the activity of complex protein structure which transports  $\beta$ -catenin to the proteasome, and results in  $\beta$ -catenin accumulation in the cytosol.  $\beta$ -catenin is then translocated to the nucleus where it regulates transcription.

Ephrin (Eph) B4 is expressed on osteoblasts and signals through RhoA GTPase, while EphB2 expressed on osteoclasts signals through PDZ proteins. Interaction of EphB2 and EphB4 couples bone resorption to formation.

Osteoclast differentiation is driven by several transcription factors, including nuclear factor of activated T-cells (NFATc1), Jun and Fos proteins, and NF $\kappa$ B. Receptor activator of nuclear factor (NF)  $\kappa$ B (RANK) is a receptor from the tumor necrosis factor receptor (TNFR) superfamily, which is essential for osteoclastogenesis. Binding of its ligand (RANKL)

activates various signaling pathways including NFκB, MAPK, c-Jun amino-terminal kinase (JNK), phospholipase Cγ (PLCγ), and phosphatidylinositol-3-kinase (PI3K). PI3K signaling links the RANK signaling pathway to the Akt proliferation and survival pathway. Binding of monocyte–colony stimulating factor (M-CSF) to its receptor (c-Fms) is essential for proliferation and survival of osteoclast precursors, and signals via PI3K/Akt and ERK.

Additionally, signaling through the immunoglobulin (Ig)-like receptor, involving DAP12 protein or FcRγ activates PLCγ, and induces calcium release, and transcription of osteoclast genes via calmodulin (CaM) and calcineurin.

Yellow arrows point to the components of osteoblast and osteoclast differentiation pathways, which are proposed to be regulated by Fas signaling.

