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Secreted Frizzled-related proteins (sFRPs) in osteo-articular diseases: much more than simple antagonists of Wnt signaling?

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Running title: sFRPs are far more than simple Wnt antagonists

Abbreviations list : adenomatous polyposis coli: APC; a disintegrin and metalloproteinases (with Thrombospondin motifs): ADAM(T)s; bone mineral content: BMC; bone mineral density: BMD; bone morphogenetic protein: BMP; Ca²⁺/calmodulin-dependent protein kinase II: CamKII; casein kinase 1: CK1; cystein-rich domain: CRD; dishevelled associated activator of morphogenesis 1: DAAM1; Dickkopfs: DKKs; extracellular matrix: ECM; enhancer of Zeste homologue: EZH2; epithelial to mesenchymal transition: EMT; fibroblast-like synoviocytes: FLS; Frizzled receptor: Fz; glycogen synthase kinase 3β: GSK3β; human adipose tissue-derived mesenchymal stem cells: hAMSCs; human mesenchymal stem cells: hMSC; lymphoid enhancer-binding factor: LEF; low-density lipoprotein receptor-related protein: LRP; matrix metalloproteinase: MMP; mesenchymal stem cells: MSC; Netrin-like domain: NTR; nuclear factor of activated T-cells: NFAT; osteoarthritis: OA; osteogenic sarcoma: OGS; phospholipase C: PLC; planar cell polarity: PCP; protein kinase C : PKC; RANK(L) receptor activator of nuclear factor kappa-B (ligand); retinoic acid receptor: RAR; RAR-related orphan receptor: ROR; rheumatoid arthritis: RA; secreted Frizzled-related proteins: sFRPs; T-cell factor: TCF; transforming growth factor: TGF; tumor necrosis factor: TNF; T regulatory cells: Treg; vascular endothelial growth factor: VEGF; Wingless-related integration site: Wnt; Wnt inhibitory factor: WIF.

Keywords: secreted Frizzled-related proteins, Wnt, inflammation, osteo-articular diseases

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Abstract

Osteo-articular diseases are characterized by a dysregulation of joint and/or bone homeostasis. These include diseases affecting originally the joints, such as osteoarthritis and rheumatoid arthritis, or the bone, such as osteoporosis. Inflammation and the involvement of Wingless-related integration site (Wnt) signaling pathways are key pathophysiological features of these diseases resulting in tissue degradation by matrix degrading enzymes, namely matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with Thrombospondin motifs (ADAMTs), secreted by the joint resident cells and/or by infiltrating immune cells. Activation of Wnt signaling pathways is modulated by different family of proteins, including Dickkopfs and the secreted Frizzled-related proteins (sFRPs). The sFRPs family is composed of five secreted glycoproteins in mammals that regulate Wnt signaling in the extracellular compartment. Indeed, sFRPs are able to bind both to the soluble Wnt ligands and to their cell membrane receptors, the Frizzled proteins. Their expression profile is altered in osteo-articular diseases, suggesting that they could account for abnormal Wnt pathways activation. In the present article, we review how sFRPs are more than simple antagonists of the Wnt signaling pathways, and discuss their pathophysiological relevance in the context of osteo-articular diseases. We detail their Wnt-dependent and their Wnt-independent roles, with particular highlights on their ability to modulate the inflammatory response and the ECM remodelling. We also discuss their potential therapeutic use with a focus on bone remodelling, osteo-articular cancers and tissue engineering.

Introduction

Osteo-articular diseases are characterised by a dysregulation of joint and/or bone homeostasis. These include diseases affecting originally the joints, such as rheumatoid arthritis (RA), spondyloarthritis and many others, or the bone, such as osteoporosis. However, it remains difficult to separate joint from bone metabolism since both are often closely entangled. This is best exemplified by osteoarthritis (OA), which has been long-term viewed as a cartilage disease and is now considered as a disease of the whole joint including major bony changes. This is also the case for osteoporosis that can occur secondarily in the context of systemic inflammation, often referred as to "inflammatory bone loss".

Historically, OA was described as a cartilage disease, but it has now been established that OA is a pathology of the whole joint. OA is an osteo-articular disease characterised by a degradation of articular cartilage and thickening of subchondral bone, with a variable level of synovial inflammation and osteophytosis[1,2] (Figure 1). A biomechanical or biochemical stress can modify the secretion profile of chondrocytes (release of pro-inflammatory cytokines, imbalance of the synthesis of extracellular matrix (ECM) components). This loss of homeostasis will also alter the behaviour of osteoblasts, osteocytes and osteoclasts, thus inducing a bone sclerosis and hypomineralization. The synovium will undergo an inflammatory process whose intensity and timing are variable. Moreover, microcracks in joints were also described as activating angiogenesis, therefore promoting the exposure of the subchondral bone to the different pathological soluble factors[3,4]. Cartilage degradation is mainly due to an increase of catabolic factors such as metalloproteinases (MMPs, most particularly MMP13) and a disintegrin and metalloproteinase with Thrombospondin motifs (ADAMTs)[5].

RA is an autoimmune disease, where synovium organizes into an aggressive inflammatory pannus containing a lot of activated immune cells and secreting huge amounts of pro-inflammatory molecules. These can induce cartilage degradation and bone erosions due to

ECM degradation by MMPs[6] (Figure 1). Osteoporosis is characterised by a loss of bone mass and a deterioration of bone microarchitecture leading to bone frailty and increased fracture risk. It occurs when there is an imbalance between bone formation and bone resorption, with more resorption than formation[7] (Figure 1).

A common feature between these osteo-articular diseases is inflammation[8] and involvement of the Wnt signaling pathways[9] among others. Indeed, these pathways play an important role in bone and joint pathophysiology by directly affecting bone, cartilage and synovial tissue homeostasis[10], but also bone mineral density (BMD)[11]. For example, the Wnt/ β -catenin pathway has been demonstrated to induce either directly or indirectly the expression of OA-associated markers (MMP13, type X collagen, ADAMTs, VEGF[9,12]. In RA, upregulation of the Wnts antagonists Dickkopf (DKK)1-3, or secreted Frizzled-related protein (sFRP)1, 2 or 4 are associated with bone erosion, particularly for sFRP1 and DKK1. Wnt/ β -catenin signaling has also been shown as enhancing the differentiation of mesenchymal stem cells in osteoblasts, and has repressing the chondrogenic and adipogenic differentiation[13]. Furthermore, Wnts downregulate bone resorption/osteoclast activation by triggering the secretion of osteoprotegerin, a natural antagonist of the interaction between receptor activator of nuclear factor kappa-B (RANK) and RANK ligand (RANKL) [9].

Two types of Wnt pathways have been described: the canonical (Figure 2a) and the non-canonical (Figure 2b) pathways. The canonical pathway is β -catenin dependent (also named Wnt/ β -catenin pathway)[10], whereas the non-canonical pathways include Ca^{2+} -dependent signaling with activation of Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) and planar cell polarity (PCP)-dependent signaling[14].

In pathological conditions, activation of the Wnt signaling pathways could be inhibited or modulated by Wnt antagonists such as DKKs and sclerostin for the canonical pathways and Wnt inhibitory Factor (WIF) and sFRPs for both canonical and non-canonical pathways[9].

How do sFRPs interact with Wnt signaling pathways?

sFRPs: a heterogeneous family of secreted proteins

The secreted Frizzled-related proteins (sFRPs) family is composed of five secreted glycoproteins in mammals (sFRP1, sFRP2, FRZB/sFRP3, sFRP4, sFRP5) that play a role as extracellular signaling ligands. This includes two closely related sub-groups: sFRP1, sFRP2 and sFRP5 form the first one, that are encoded by three exons on human chromosome 8p11.21, 4q31.3 and 10q24.2, respectively; FRZB/sFRP3 (Frizzled motif associated with bone development) and sFRP4 form the second one, that are encoded by six exons on chromosome 2q32.1 and 7p14.1, respectively[15]. The third sub-group has been identified in *Xenopus*, zebrafish and chick, but not in mammals[16]. It is composed of Sizzled, Sizzled2 and Crescent[17]. They were first described as antagonists of the Wnt/ β -catenin pathway during embryonic development[18].

Each sFRP is around 300 amino acids in length, and is structured with a N-terminal cysteine-rich domain (CRD) and a C-terminal Netrin (NTR)-like domain (Figure 3). The CRD domain shares 30-50% sequence homology with the CRD of Frizzled (Fz) receptors[16,19]. Expression of sFRPs is altered in various diseases including cancers, retinal degeneration, hypophosphatemic diseases, but also in osteoarticular and rheumatic diseases which underlines that their activity is essential for tissue homeostasis[20,21].

Depending on the tissue and on the life stage, the expression profile of sFRPs varies. Indeed, each sFRP is not expressed in the same way in the different tissues composing the joint (Table 1).

sFRPs are developmentally regulated proteins able to modulate Wnt signals

The first sFRP, FRZB, was discovered in 1996 by Hoang and colleagues[22], in articular cartilage extracts prepared from newborn calves[23]. *FRZB* was expressed in the cartilaginous areas of developing long bones during fetal development (between 6 and 13 weeks). This suggested the important role of FRZB/sFRP3 in the morphogenesis of mammalian skeleton[22].

The role of FRZB was further investigated in 1997 in the Spemann organizer of *Xenopus*[24]. Spemann organizer is a dorsal region with a small group of cells in early vertebrate embryos, inducing the formation of neural tissues and the dorsalization of ventral mesoderm leading to the formation of somites[17]. In this study, Leyns and colleagues showed that FRZB was able to bind to and block Wnt-8 signaling, thus demonstrating that it was a Wnt antagonist in *Xenopus*[24]. The same year, Wang and colleagues isolated FRZB to study its axial patterning in *Xenopus* embryos and its biological activities during gastrulation[25]. They confirmed the inhibitory activity of FRZB on Wnt-8 to prevent an inappropriate ventral signaling in developing dorsal tissues[25]. At the same time, Rattner and colleagues identified sFRPs in mice and rats and described their interaction with *Drosophila* Wingless, presumably *via* their CRD domain[19]. In parallel, Mayr and colleagues cloned a sFRP called Fritz, a mouse (*mfiz*) and a human (*hfiz*) gene encoding a secreted protein structurally related to the extracellular portion of the *Drosophila* and vertebrate *frizzled* genes[26]. Fritz has been found in all anatomical sites where Wnt proteins were previously involved in the induction of developmental processes. The authors demonstrated the inhibitory role of Fritz on Wnt biological activities, when both proteins were ectopically expressed in *Xenopus* embryos. sFRPs are also able to interfere with genetic protein (BMP) signaling. Indeed, Ellies and colleagues showed that overexpression of sFRP2 inhibited BMP4 expression in the rhombomeres 3 and 5 by antagonizing Wnt signaling, thus preventing programmed cell death physiologically occurring during the loss of premigratory neural crest cells from rhombomeres 3 and 5 in the avian hindbrain[27].

It is important to underline that sFRPs are able to bind both to soluble Wnt proteins in the extracellular compartment and to membrane Frizzled (Fz) receptors, and this capacity directly affects the outcome of the Wnt signaling milieu[28]. Indeed, sFRP1 was shown to potentiate the Wnt3a-dependent activation of the β -catenin pathway when Fz5 was overexpressed at the surface of L929 fibroblasts. However, sFRP1 inhibited this pathway when Fz2 was overexpressed[28].

Altogether, these elements support that sFRPs can antagonize Wnt signaling by at least two different ways: i) the tethering of Wnt proteins in the extracellular space independently of any binding to Fz receptors (Figure 4, ①); ii) the binding to Fz receptors masking the Wnt binding site by steric hinderance (Figure 4, ②). However, the Wnt- β -catenin pathway can also be activated in the presence of sFRPs if there is a co-fixation of the sFRP and the Wnt ligand on the Fz receptor[29] (Figure 4).

Alternatively, Mii and Taira demonstrated that sFRPs were able to expand Wnt signaling range[30]. Wnts and sFRPs are involved in the embryonic development, and the authors studied the gradient of Wnt ligands (posterior) and sFRPs proteins (anterior) during the formation of the antero-posterior axis in the *Xenopus* embryos. They observed that in presence of sFRPs, Wnt signal was extended in the anterior part, whereas this extension of the signal was not observed without sFRPs. To extend this signal, Wnts-sFRPs complexes were formed. Thus, sFRPs could also act as Wnt transporters to enhance their signal[30].

Recapitulation of a developmental process during osteo-articular pathologies

Wnts and sFRPs are involved in endochondral ossification, which is the transition of cartilage to bone. This is a physiological process that occurs during embryonic development in long bone formation, and postnatally in the growth plate[31]. Endochondral ossification is also involved in enthesis formation, which is the point of insertion of a tendon, ligament, joint capsule or fascia to bone[32]. Briefly, endochondral bone formation is a sequential process by which chondrocytes will differentiate through different stages. Resting chondrocytes will turn into proliferative chondrocytes under the influence of different secreted factors, including Wnts and BMPs. Cells will then transit through a pre-hypertrophic stage, characterized by chondrocytes secreting high levels of indian hedgehog. Finally, these cells will differentiate into hypertrophic chondrocytes, which express type X collagen and MMP13. Finally, hypertrophic chondrocytes will undergo apoptosis, which will result in the cartilage ECM breakdown, followed by an invading ossification front (penetration of blood vessels, osteoclasts and osteoblastic progenitors)[33]. In the pathophysiology of OA, there is a recapitulation of this developmental program (endochondral ossification)[34], which induces the progression of the phenotype of articular chondrocytes towards hypertrophy[35]. Also, new bone formation is observed during enthesitis, a characteristic feature found in spondyloarthritis[36]. Therefore, sFRPs are likely to be tightly associated to the onset and or to the progression of such osteo-articular diseases.

sFRPs play Wnt-independent roles

sFRPs can act as proteinase inhibitors in retinal neurogenesis, as sFRP1 and sFRP2 were shown to inhibit ADAM10[37], with the subsequent loss of Notch signaling activation. In 2015, a team found a new role for FRZB/sFRP3, that consists in the suppression of the enzymatic activity of ADAM17 responsible for IL-6R shedding[38]. ADAM17, also called TACE, plays a role in arthritis by shedding membrane-anchored molecules or receptors, such as tumor necrosis factor (TNF) α and IL-6R, to release them into the synovial fluids. The soluble form of IL-6R, sIL-6R, is associated with pathological inflammatory responses due to its ability to signal upon binding with its transmembrane transducer glycoprotein 130[39]. The study demonstrated that FRZB/sFRP3 was able to reduce the sheddase activity of ADAM17 towards IL-6R, but also TNF α and transforming growth factor (TGF) α , by approximately 30%. Noteworthy, the authors showed that the rare double variant of FRZB/sFRP3, R200W-R324G, which is associated with an increased risk of OA, was neither able to interact with nor to suppress ADAM17 activity (Figure 5).

Moreover, sFRP1 has a high affinity for a particular motif: L/V-VDGRW-L/V. This motif is present on two known proteins: UNC5H3 playing a repulsive role during axonal guidance, and receptor activator of nuclear factor kappa-B Ligand (RANKL), involved in the activation of osteoclasts[40,41].

sFRPs and the control of the immune/inflammatory response

sFRP1 promotes Th17 differentiation

RA is a chronic inflammatory disease inducing the articular cartilage destruction and bone resorption. This disease involves an interplay between T and B cells, macrophages and fibroblast-like synoviocytes (FLS) [42]. Th17 cells were initially described in the response to exogenous bacteria and fungi, but have also been shown as playing a role in several inflammatory diseases. In such pathologies, T regulatory cells (Tregs) are modulators of the immune response, In RA, there is a deregulation in the balance between Th17 and Tregs in favour of Th17. It has been demonstrated that IL-17 secreted by Th17 increased the secretion of pro-inflammatory cytokines and prostaglandins, activated osteoclastogenesis, induced

pannus growth (thickening of the synovium) and also favoured neoangiogenesis through increased vascular endothelial growth factor (VEGF) production [43]. Lee and colleagues [44] showed in 2012 that sFRP1 was able to promote Th17 differentiation in rheumatoid arthritis (RA). They reported first that the concentration of both sFRP1 and IL-17 were higher in the synovial fluid of patients with RA than in patients with osteoarthritis (OA). Moreover, they investigated the effects of sFRP1 on human naïve CD4⁺ CD45RA⁺ T cells from healthy controls stimulated with anti-CD3 and anti-CD28. They showed that sFRP1 stimulated IL-17 production by T cells in a dose-dependent manner[44].

Physiologically, estrogens regulate osteoclast differentiation via IL-17 blockade. IL-17 can activate osteoclasts differentiation by increasing the production of pro-osteoclastogenic cytokines including TNF- α , IL-6 and RANKL. In case of estrogens deficiency, for example in post-menopausal women, Th17 cells differentiation and IL-17 production increased, thus contributing to bone loss. Therefore, secondary to estrogens depletion, Th17 are implicated in bone resorption [45]. Likewise, the parathyroid hormone (PTH) is an important regulator of serum and urinary calcium and phosphorus levels. An excessive production of PTH is associated with an increased bone loss, as PTH is described as being able to induce Th17 cells proliferation and IL-17 production. This results in an increased sensitivity of osteoblasts and osteocytes to PTH, and triggers RANKL secretion[46].

The role of Th17 in joint non physiological conditions is much less clear. In bone, they can contribute to bone regeneration by activating mesenchymal stem cells during fracture healing[47].

The Wnt/ β -catenin pathway, and particularly TCF1, is involved in the repression of the IL17 locus during thymic T-cell development, which controls Th17 differentiation[48]. By acting as a Wnt/ β -catenin inhibitor, sFRP1 is able to favour Th17 differentiation, as shown by Lee and colleagues [44]. In addition, sFRP1 has been shown to increase the sensitivity of naïve CD4⁺ T cells to TGF- β , as illustrated by the enhanced Smad 2 and Smad 3 phosphorylation[44]. Interestingly, the link between activation of the TGF- β pathway and Th17 differentiation has been recently highlighted[49]. Indeed, the authors showed that the number of Th17-producing cells was reduced in Smad 2 knockout mice compared to WT littermates, indicating that Smad 2 is required for Th17 cell generation[49].

sFRP1 has also the capacity to up-regulate the retinoic acid receptor (RAR)-related orphan receptor C (RORC) gene[44]. RORC codes for the DNA-binding transcription factor ROR γ that belongs to the NR1 subfamily of nuclear hormone receptors. ROR γ is a one of the pivotal transcription factor driving Th17 differentiation and IL-17A production in mammals. Its activation has also been associated with a decreased IL-1R1 expression, which is critical for Th17 differentiation[50] (Figure 6).

In summary, sFRP1 seems to be a key player in the pathophysiology of RA. Targeting sFRP1 could be of interest to manage the enhanced Th17 differentiation occurring in the disease, when anti-TNF α biotherapies fail or stop being effective.

sFRPs are epigenetically regulated proteins limiting synovitis by down-regulating fibronectin

Among joint resident cells, fibroblast-like synoviocytes (FLS) play a pathophysiological role in RA by contributing to inflammation and tissue degradation through the secretion inflammatory mediators (prostaglandins, nitric oxide, cytokines) and matrix-degrading enzymes[51]. Wnt/ β -catenin is one of the signaling pathways that have been shown to be contributive to FLS activation. FRZB/sFRP3 and DKK1 are the most described negative

regulators of this pathway, which also contributes to several critical aspects of joint pathophysiology.

In 1997, Elhaj Mahmoud and colleagues studied the effects of FRZB/sFRP3 and DKK1 on RA FLS derived from synovial fluids[52]. They demonstrated that FRZB/sFRP3 down-regulated fibronectin expression by decreasing the activation of Wnt/ β -catenin pathway in these cells. In 2002, Sen and colleagues showed that sFRP1 was also able to down-regulate fibronectin expression in RA FLS and that it reduced cell survival[53]. Fibronectin modulates joint inflammation through chemotactic properties. It usually enhances FLS survival and facilitates adhesion of FLS and macrophages on cartilage surface[42,54]. In 1999, Sarkissian and colleagues showed that fibronectin was also capable to promote FLS proliferation[55]. Moreover, Vartio and colleagues reported in 2008 that the production of fibronectin, assessed by immunofluorescence staining, was increased in the synovial membrane from RA patients compared to healthy patients. Taken together, adherence and proliferation of FLS and macrophages in the joint stimulate the secretion of pro-inflammatory factors in the cartilage, thus promoting its degradation[56].

Moreover, the synthesis of fibronectin by FLS was shown to promote the migration and retention of mesenchymal stem cells and leukocytes in RA synovium, supporting that sFRPs may be anti-inflammatory in this context (Figure 7).

An epigenetic repression of sFRP1 was described recently in RA patients[57]. The authors demonstrated that enhancer of Zeste homologue (EZH2), a histone-lysine N-methyltransferase enzyme that was overexpressed in FLS, down-regulated the expression of its direct target gene sFRP1. Such overexpression of EZH2 was explained by the inflammatory environment occurring in RA and sFRP1 silencing was ascribed to addition of three methyl groups to H3K27 (histone 3 lysine 27)[57].

Taken together, these data support that sFRPs expression is epigenetically regulated in inflammatory synovium and that any decrease of sFRPs levels may contribute to the self-perpetuation of joint inflammation.

sFRPs regulate the progression of osteoporosis-related features

Osteoclasts and osteoblasts are critical cells for bone remodelling. Osteoblasts are responsible for bone formation and mineralization, whereas osteoclasts account for bone resorption[58]. Bone homeostasis is the result of their coordinated activities since osteoblasts also regulate osteoclasts activation by secreting several factors, such as interleukins, RANKL and Macrophage-Colony Stimulating Factor. An imbalance in the production of mediators regulating bone turnover results in pathologies like osteoporosis, which is a disease characterised by a change in the bone microarchitecture and by a loss of bone mass density[59].

In 2004, Häusler and colleagues showed that sFRP1, expressed and secreted by osteoblasts, was able to inhibit osteoclast formation by a direct binding to RANKL, a mechanism mimicking those of osteoprotegerin [40]. Indeed, the complexation of RANKL by sFRP1 will prevent its fixation on the RANK receptor, which is expressed on osteoclasts precursors and necessary for their final differentiation and activation. As the Wnt/ β -catenin pathway is known to positively regulate bone mass and strength[13], it seems paradoxical that a Wnt antagonist could have a similar effect by inhibiting osteoclast formation. However, one must underline that the later effect was supported by a binding to RANKL independently of Wnt/ β -catenin pathway (Figure 8).

Contrasting results were obtained in another study focusing on the role of miR-542-3p in bone formation. This miR, which targets the 3'-UTR of sFRP1 mRNA, was shown to prevent ovariectomy-induced osteoporosis in rats[60]. In addition to its inverse relationship with

sFRP1, it was shown that miR-542-3p mimic increased expression of the osteoblast-specific markers alkaline phosphatase, Runx-2 and osteocalcin during mesenchymal stem cells (MSC) differentiation.

However, one must underline that these two studies are not directly comparable since: i) they were not performed in equivalent models: osteoblast and osteoclast lineage challenged with sFRP1 in one hand, bone and bone marrow cells from ovariectomized rats treated with miR-542-3p inhibitor on the other hand; ii) they did not decipher the effects of sFRP1 in the same context of bone remodelling: direct effect of sFRP1 on in vitro osteoclast formation[40] in one case, indirect in vivo effect on osteoblastogenesis in the other case[61].

Another study found a new epigenetic regulation of sFRP1 by the Histone Lysine Demethylase 7A (KDM7A) during osteogenesis. KDM7A could be able stimulate sFRP1 expression by demethylating H3K9me2 and H3K27me2 in progenitor cells[62], thus inhibiting osteogenesis. These data support that sFRP1 could contribute to osteoporosis by reducing osteoblastogenesis.

To confirm these results, Wu and colleagues have down-regulated sFRP1 expression by using a miR-27a to study the reosseointegration in a model of canine peri-implantitis. Down-regulation of sFRP1 induced a better bone formation and reosseointegration[63].

Thus, sFRP1 could have a variable impact on bone density, depending possibly on the differentiation stage of the cell populations and the variable contribution of Wnt independent mechanisms.

A study established a link between single nucleotide polymorphisms in sFRP1 and bone mineral density (BMD) and/or bone mineral content (BMC)[64] in a population of osteoporotic patients. The authors analysed the presence of rs1127379 (c.1406A>G) and of rs3242 (c.3132C>T) variants in the 3'UTR of sFRP1 gene as possible predisposition factors. They reported that the c.[1406A; 3132C] haplotype was associated with a lower lumbar spine BMD and BMC, and that the c.[1406G ; 3132C] haplotype was associated with a lower lumbar spine BMC.

Moreover, in another study, a variant of the FRZB/sFRP3 gene (R200W-R324G haplotype) has been associated with osteolysis. The authors suggested that this was caused by a decrease in its ability to antagonize Wnt signaling[65]. This possibility has also been raised in the work of Thyssen and colleagues in 2016, where the stable overexpression of FRZB/sFRP3 in the osteogenic cell line MC3T3-E1 increased osteogenesis while decreasing Wnt/ β -catenin pathway activation[1].

In 2016, a team hypothesized that an autosomal recessive disorder of long bone and wide metaphyses named Pyle's disease was associated with a mutation in *sFRP4*. They generated sFRP4 knockout mice, which reproduced the symptoms (cortical-bone defects) of Pyle's disease patients. The authors explained this result by a dysregulation in the Wnt and BMP signaling pathways and demonstrated that the treatment of mice with a soluble BMP-2 receptor or with anti-sclerostin antibodies improved bone disorders[66]. Interestingly, in 2017, another team found a human mutation in *sFRP4* (c.183C>G) that was associated with Pyle's disease[67].

Thus, sFRPs are playing an important role in the regulation of bone pathophysiology, especially concerning calcification/mineralization of the extracellular matrix/bone mineral content.

sFRPs and the control of cartilage homeostasis

The Wnt/ β -catenin pathway has been extensively demonstrated to actively contribute to the development of OA. Indeed, the activation of this pathway cause cartilage degradation with the modification of chondrocytes phenotype, turning them hypertrophic [9].

In 2014, Takamatsu and colleagues studied the effects of Verapamil, a molecule capable of downregulating Wnt/ β -catenin signaling by triggering the overexpression of FRZB/sFRP3. They showed that OA progression was inhibited after treatment with Verapamil[68].

sFRPs are tightly involved in the control of cartilage development

Physiologically, sFRPs have a role during embryogenesis. sFRP1 is involved in limbs growth, and is expressed by proliferating and hypertrophic chondrocytes at E15.5[69]. sFRP2 expression has been found in the limb mesenchyme and synovial joints[70]. Physiologically, FRZB/sFRP3 is expressed during chondrogenesis in the early limb mesenchyme in the chick., In mice, FRZB/sFRP3 expression has also been found persisting in the pre-hypertrophic and hypertrophic chondrocytes, and in the periosteum at E15.5[69]. sFRP4 has been detected in hypertrophic chondrocytes areas[69].

FRZB/sFRP3 prevents articular cartilage degradation

In 2014, Bougault and colleagues demonstrated a protective role of FRZB/sFRP3 on the degradation of articular cartilage ECM[71]. Indeed, using explants from FRZB/sFRP3^{-/-} mice, they showed that GAG release and global MMP activity induced by mechanical loading were increased in comparison to wild-type controls. Moreover, MMP3 and MMP13 expression and protein release were higher in primary chondrocytes from FRZB/sFRP3^{-/-} mice compared to wild-type animals after stimulation with IL-1 β [71]. In both case, the enhanced catabolic response was associated with an over-stimulation of the canonical Wnt/ β -catenin pathway. These data demonstrate that FRZB/sFRP3 plays an important role in the prevention of cartilage degradation by attenuating the deleterious effects of canonical Wnt/ β -catenin pathway activation. Cartilage degradation is a main feature of OA, and FRZB/sFRP3 has been strongly linked to the progression and onset of OA.

Lack or decrease of FRZB/sFRP3 is associated with OA or OA-related changes

In 2004, Loughlin and colleagues associated functional variants of FRZB/sFRP3 with susceptibility to hip OA in women, as they demonstrated that mutated forms had a reduced the ability of FRZB/sFRP3 to oppose the Wnt/ β -catenin pathway[72]. These FRZB variants are mutated on highly conserved arginine residues in exon 4 with a substitution C \rightarrow T (R200W) and a substitution C \rightarrow G (R324G) respectively.

Moreover, this double variant was also used to demonstrate Wnt-independent properties of FRZB/sFRP3. Indeed, unlike the wild-type FRZB/sFRP3, the double variant was unable to suppress the enzymatic activity of ADAM17, responsible for IL-6R shedding[38].

Moreover, FRZB/sFRP3 knock-out mice showed an increase of Wnt/ β -catenin pathway activation, as well as of MMP3, at the transcriptome level[73]. The role of FRZB/sFRP3 has also been highlighted in vitro, where an overexpression of FRZB/sFRP3 induced an increase of the phenotypic markers of the healthy articular chondrocytes[73].

It has also been shown that the expression of FRZB/sFRP3 is down-regulated in OA, and that a decrease of inhibitor of the canonical Wnt/ β -catenin pathway is associated with an increased MMP13 expression[74].

OA is also characterized by a variable level of inflammation. IL-1 β is a pro-inflammatory cytokine with a pathophysiological role in OA due to its ability to promote the secretion of cartilage degrading enzymes as well as the release of pro-inflammatory mediators including cytokines, prostaglandins and nitric oxide[75]. Interestingly, a crosstalk between Wnt/ β -catenin and IL-1 β signaling pathways has been described[76]. Indeed, IL-1 β , *via* the

stimulated synthesis of NO, was able to downregulate the expression of the Wnt antagonists DKK1 and FZB/sFRP3 in OA cartilage.

Altogether, these elements highlight the importance of FRZB/sFRP3 in cartilage protection in the context of OA, mainly through Wnt/ β -catenin pathway inhibition, but also via a proteinase-mediated Wnt-independent effect.

sFRPs as potential therapeutic options?

Modulation of sFRP1 to promote bone remodelling?

sFRP1 has negative effects on bone formation and osteoblasts survival and promotes osteoporosis. A strategy for the treatment of osteoporosis could consist in the use of sFRP1 inhibitors. Shi and colleagues developed imino-oxothiazolidine derivatives, and one of these showed encouraging results in *ex vivo* cultures of mouse calvaria. Indeed, the compound increased bone formation and the number of osteoblasts in comparison with untreated control[77].

Gopalsamy and colleagues proposed to use Diarylsulfone Sulfonamides to inhibit sFRP1[78]. They synthesized 13 compounds with different chemical modifications by introducing small substituents (methyl, O-methyl, fluoro, ethyl, hydrogen). One of the compounds increased bone formation and osteoblasts number in *ex vivo* cultures of mouse calvaria[78]. Based on this work, the team developed a N-Substituted Piperidinyl Diphenylsulfonyl Sulfonamide (WAY-316606) and found a similar effect on bone formation and osteoblast activation *ex vivo* in mouse calvaria[79]. Interestingly, WAY-316606 was tested in a TCF luciferase gene reporter system of cells stimulated with a combination of Wnt3a and sFRP1. This compound induced a 9-fold increase in luminescence when used at 0.65 μ M[80]. WAY-316606 was also able to increase bone formation in the *ex vivo* mouse calvaria model in a dose dependent manner, with an EC₅₀ of 1nM.

All these studies on sFRP1 inhibitors allowed the discovery of potential new molecules for the treatment of osteoporosis.

Using sFRPs in cancer therapy?

Several studies showed an effect of sFRPs in chondrosarcoma invasion and metastasis. Sheng and colleagues found that sFRP5 expression was submitted to an epigenetic regulation in chondrosarcoma cell lines. Indeed, CpG islands of the sFRP5 promoter were hypermethylated by DNA methyltransferases inducing a decrease of sFRP5 expression[81]. This downregulation of sFRP5 in chondrosarcoma cell lines increased cell invasion and metastasis potency through activation of the Wnt5a/ β -catenin pathway.

Others studies showed the involvement of FRZB/sFRP3 in osteosarcoma. In 2007, Mandal and colleagues studied FRZB/sFRP3 expression in osteogenic sarcoma (OGS) cell lines and OGS biopsy specimens in comparison with immortalized fetal osteoblasts and normal bone specimens. FRZB/sFRP3 expression was down-regulated in OGS biopsy specimens compared to normal bone specimens, and was almost completely lost in OGS cell lines compared to fetal osteoblasts. Karyotype abnormalities, such as multiplications and translocations, were detected on chromosome 2 where FRZB/sFRP3 gene is located, and this observation could be one of the explanation for the down-regulation of FRZB/sFRP3[82].

In 2017, Bravo and colleagues showed the effects of the anti-tumor 2-methoxyestradiol (2-ME) on FRZB/sFRP3 in osteosarcoma cells. 2-ME was able to activate the promoter of FRZB/sFRP3, thus triggering the subsequent gene transcription, leading to increased proteins levels. Moreover, autophagy and apoptosis induced by 2-ME was decreased by FRZB/sFRP3

siRNA. Altogether, this suggested the potential use of FRZB/sFRP3 in osteosarcoma[83]. In 2018, the same team analysed the sera of 67 osteosarcoma and age-matched non-disease controls, and observed that the level of FRZB/sFRP3 was lower in osteosarcoma samples. Moreover, immunohistochemical analysis showed a decrease of FRZB/sFRP3 expression in osteosarcoma tissues. RNA sequencing performed on osteosarcoma and control tissues revealed that the reduced FRZB/sFRP3 expression was concomitant to the expression of several Wnt pathways members [20]. These results indicate that FRZB/sFRP3 could be important for the diagnosis and the blockade of osteosarcoma progression.

Lin and colleagues studied the role of sFRP1 in osteosarcoma invasion[84]. They overexpressed a miR-940 mimic, which targeted the 3'UTR of sFRP1 mRNA. miR-940 up-regulation decreased sFRP1 expression, and induced an increase of osteosarcoma cells proliferation, invasion and tumor growth[84]. Thus, inhibiting miR-940 or using recombinant sFRP1 in osteosarcoma cells could reduce the tumor progression and metastasis.

In contrast, Kim and colleagues studied the effect of sFRP2 overexpression in patients with the Li-Fraumeni syndrome, which is characterised by a p53 mutation and an increased incidence of osteosarcoma (500 fold higher in comparison to the normal population)[85]. sFRP2 is naturally overexpressed in patients with Li-Fraumeni syndrome, and in osteosarcoma cell lines with a p53 mutation. Moreover, sFRP2 overexpression induced an abnormal differentiation of pre-osteoblasts and induced pluripotent stem cells-derived osteoblasts from patients with Li-Fraumeni syndrome. This abnormal differentiation of pre-osteoblasts resulted in their transformation into osteosarcoma-like cells. Overexpression of sFRP2 affected angiogenesis, a critical phenomenon in cancer invasion and metastasis, and a transcriptomic analysis revealed that sFRP2 overexpression increased the expression of pro-angiogenic and anti-apoptotic genes in endothelial cells[85]. Consistent with these results, Techavichit and colleagues showed the implication of sFRP2 in invasion and metastasis[86].

Bhuvanalakshmi and colleagues have established breast cancer stem-like cells as a chemo-resistant and tumor-initiating population. Their goal was to eradicate this population, significantly improving patients survival, by using Diosgenin, a steroidal saponin [87]. The mechanism of action of Diosgenin turned out to up-regulate the expression of sFRP4. This resulted in a down-regulation of β -catenin, which repressed the effectors of epithelial-mesenchymal transition and pro-invasive markers[87] (Figure 9).

The same team has worked on glioma stem-like cells. They demonstrated that sFRP4 was able to chemo-sensitize these cells to the anti-glioblastoma drug, temozolomide, in correlation with the down-regulation of drug resistance markers (ABCG2, ABCC2, ABCC4). Alike their study on breast cancer stem-like cells, they reported an inhibition of EMT, accompanied by a decrease of several mesenchymal markers (Twist, Snail, N-cadherin) and an increase of the epithelial marker E-cadherin[88] (Figure 9).

In 2018, Zeng and colleagues explore the ability of sFRP2 to regulate the epithelial to mesenchymal transition (EMT) in choriocarcinoma, which is a highly aggressive gestational trophoblastic neoplasia. They increased sFRP2 expression in choriocarcinoma cell lines *via* its promoter demethylation. Up-regulation of sFRP2 reversed the EMT process[89] (Figure 9).

Thus, sFRPs could be a good treatment strategy to reduce EMT inducing metastasis and to increase the drug chemo-sensitivity.

sFRPs as key factors in tissue engineering?

In 2015, Katagiri and colleagues studied the effect of FRZB/sFRP3 in bone regeneration by implanting human mesenchymal stem cells (hMSCs) into a rat calvarial bone defect in the presence or absence of FRZB/sFRP3[90]. After 4 and 8 weeks of implantation, they observed higher bone regeneration in the presence of FRZB/sFRP3 in comparison to controls. This

work suggested that FRZB/sFRP3 could be helpful for the treatment of bone defects[90]. This publication did not study the role of FRZB/sFRP3 in chondrogenesis. However, in 1996, Hoang and colleagues[22] have isolated FRZB/sFRP3 from chondrogenic extracts and hypothesised that it could play a role in skeletal morphogenesis: FRZB/sFRP3 could contribute to endochondral ossification, resulting in the growth of cartilage and to its transformation into bone. Depending on the context, this is a physiological or pathological process. Indeed, it occurs during skeletal growth[91], but can be recapitulated during OA with the transdifferentiation of hypertrophic chondrocytes into osteoblasts[92].

Park and colleagues studied the ability of sFRP4 to promote adipogenesis by human adipose tissue-derived mesenchymal stem cells (hAMSCs). They found that sFRP4 was upregulated during adipogenesis with a decrease of the Wnt/ β -catenin pathway in hAMSCs. Moreover, the use of a siRNA targeting sFRP4 decreased differentiation of hAMSCs into adipocytes, underlining the importance of sFRP4 during adipogenesis[93].

The effects of sFRP1 on chondrogenesis of hMSCs were also studied. The authors showed that sFRP1 could play a role in early chondrogenesis of hMSCs, but they did not observe a strong effect in comparison to usual factors such as TGF- β [94].

To conclude with the role of sFRPs and tissue engineering, it could be interesting to use FRZB/sFRP3 for cartilage growth and/or bone regeneration, and sFRP4 to promote adipogenesis. The role of sFRP1 in chondrogenesis would still require more investigations, as this molecule may be susceptible to fine-tune chondrogenic differentiation.

Conclusion

In summary, the role of sFRPs is critical in joint and bone homeostasis. We sum up the different described roles fulfilled by each sFRP in pathophysiological situations in Figure 10. sFRPs could represent an interesting strategy to regulate joint dysfunction because of their effects on Wnt signaling pathways and different actors of inflammatory-related signals. In the control of the inflammatory response, the reduction of sFRP1 level represents a promising research avenue to control Th17 differentiation, especially when biotherapies turn to become ineffective. Concerning bone diseases, the impact of sFRPs is more contrasted. Indeed, while the ability of sFRP1 to control osteoclastogenesis, through binding to RANKL, could be interesting in the treatment of osteoporosis, overexpression of sFRP1 was deleterious in some cases. Therefore, further researches are required to decipher this discrepancy. For example, a fine-tuning of the concentrations of sFRP1, or the search of a possible contribution of other Wnt pathways members seem key elements to better define the pathophysiological role of sFRP1 in bone remodelling. Similarly, maintaining a sufficient level of FRZB/sFRP3 within the articular cartilage seems critical to preserve the tissue homeostasis, which could help to prevent OA progression. Interestingly, sFRPs have an important role in the prevention of osteoarticular cancers, and promoting FRZB/sFRP3 expression, or using recombinant protein looks promising in osteosarcoma. The role of sFRPs in tissue engineering requires further research, but these proteins seem to have potential to fine-tune mesenchymal stem cells differentiation to achieve a stable articular chondrocyte phenotype, which remains challenging at the present time.

Author Contributions

MC wrote the manuscript and prepared the figures, J-YJ drafted the manuscript, FC drafted the manuscript and planned the review.

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Figures and table legends

Figure 1: Osteoarthritis is characterised by cartilage degradation by MMPs and ADAMTs, abnormal cellular proliferation in the synovium and bone sclerosis and hypomineralisation of the subchondral bone. Rheumatoid arthritis is characterised by bone erosion and inflammation of the synovial membrane and osteoporosis by loss of bone mass, modification of the bone structure and increased bone resorption.

Figure 2a: Wnt canonical pathway. Without binding of Wnt ligand to a member of the G protein-coupled receptor family Frizzled (Fz), β -catenin is phosphorylated by a protein complex composed of Axin, CK1 (casein kinase 1), APC (adenomatous polyposis coli) and GSK3 β (glycogen synthase kinase 3 β). This leads to β -catenin ubiquitination and then degradation by the proteasome. When Wnt ligand binds to its receptor, the Fz co-receptor low-density lipoprotein receptor-related protein (LRP)5/6 is recruited and Dishevelled is activated, thus inhibiting β -catenin phosphorylation. In turn, β -catenin is translocated into the nucleus and activates gene transcription with co-transcription factors T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF).

Figure 2b: Non-canonical Wnt pathways. The Wnt/Ca²⁺ pathway is triggered when the binding of Wnt to a member of the G protein-coupled receptor family Frizzled (Fz) stimulates phospholipase C (PLC) through dishevelled activation. This induces an intracellular Ca²⁺ release, which activates protein kinase C (PKC) and Ca²⁺/calmodulin-dependent protein kinase II (CamKII). These messengers will trigger nuclear factor of activated T-cells (NFAT) translocation to the nucleus to mediate transcriptional effects. PCP pathway is triggered when dishevelled recruits DAAM1 (dishevelled associated activator of morphogenesis 1), which will activate Rac or RhoA kinases.

Figure 3: General structure of sFRPs and major functional roles of each domain. The numbers in superscript indicate the references used as source information.

Figure 4: The activation status of Wnt signaling pathways depends on the combination of Wnt ligands and sFRPs.

Figure 5: Wnt-independent effects of sFRPs are mediated by ADAM inhibition and RANKL binding.

Figure 6: sFRP1 favours Th17 differentiation by Wnt-dependent and Wnt-independent mechanisms.

Figure 7: sFRP1 and FRZB/sFRP3 exert anti-inflammatory effects by reducing fibronectin expression by fibroblast-like synoviocytes.

Figure 8: RANKL, expressed on the surface or released by osteoblasts, can bind to its receptor RANK, expressed by pre-osteoclasts. This binding triggers the differentiation of pre-osteoclasts into osteoclasts, which are the main driver of bone resorption. sFRP1 is able to decrease bone resorption through its binding to RANKL.

Figure 9: Epithelial-mesenchymal transition is a process characterised by the ability of epithelial cells to lose their properties to acquire characteristics of mesenchymal stem cells. This transition permits to cancer cells to acquire invasive properties and to migrate into other tissues by intravasation and create metastasis. sFRP2 and sFRP4 could inhibit EMT by down-regulating mesenchymal markers and up-regulating epithelial markers.

Figure 10: Summary of the different described roles fulfilled by each sFRP in pathophysiological situations.

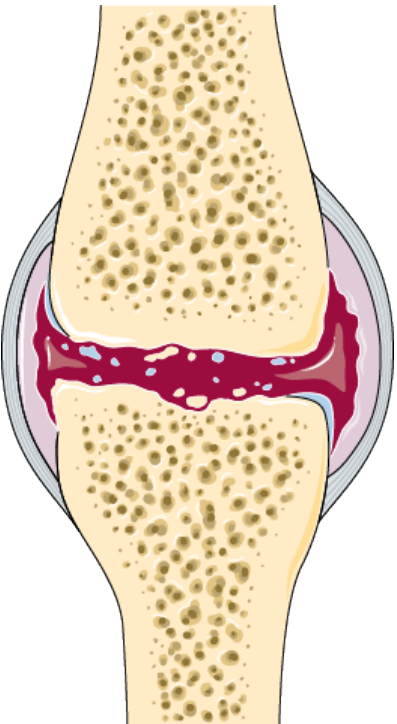
Table 1: mRNA and protein expression of sFRPs in the developing joint and in the osteoarticular tissues in basal and pathological conditions. Pathologies are abbreviated as follow, OA: osteoarthritis, RA: rheumatoid arthritis. Species are abbreviated as follow, H: human, M: mouse, R: rat, C: calf. The numbers indicate references used as source information.

TABLE 1

sFRPs	Nature	Cartilage	Diseased Cartilage	Bone	Diseased Bone	Synovium	Diseased Synovium	Developing joint
sFRP1	RNA	M [95] H [96]	H, OA [96]	M, [95,97, 98]		M [95,97]	M, RA [97,99] H, RA, OA [100]	[18,40,69,80 ,101–104]
	Protein	M [95]		M [97]		M [95,97]		[40,79,101, 103]
sFRP2	RNA			M [98]		M [97] R [105]	M, RA [97,99] R, RA [105]	[102,103, 105]
	Protein					M [97] R [105]	R, RA [105]	[104]
FRZB/ sFRP3	RNA	H [74,107, 108] C [22]	H, OA [108–110]	M [98] H [111]	H, OA [110]	M [98]	H, RA, OA [100]	[68,102, 103]
	Protein	C [22]		H [111]				
sFRP4	RNA	H[96,108]	H, OA [96,108, 112]	M [98]	H, OA [112]	M [97] R [113]	M, RA [69,97] H, RA [100] H, OA [112,113] R, RA [113]	
	Protein					M [97] R [113]	R, RA [113]	
sFRP5	RNA						H RA, OA [114]	[69]
	Protein							

Figure 1

Osteoarthritis

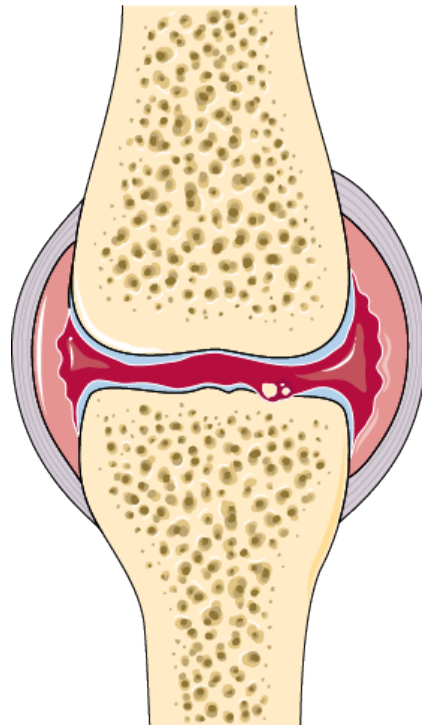


Cartilage degradation
(MMPs, ADAMs)

Synovium:
Inadequate cellular proliferation

Bone sclerosis
Hypomineralisation

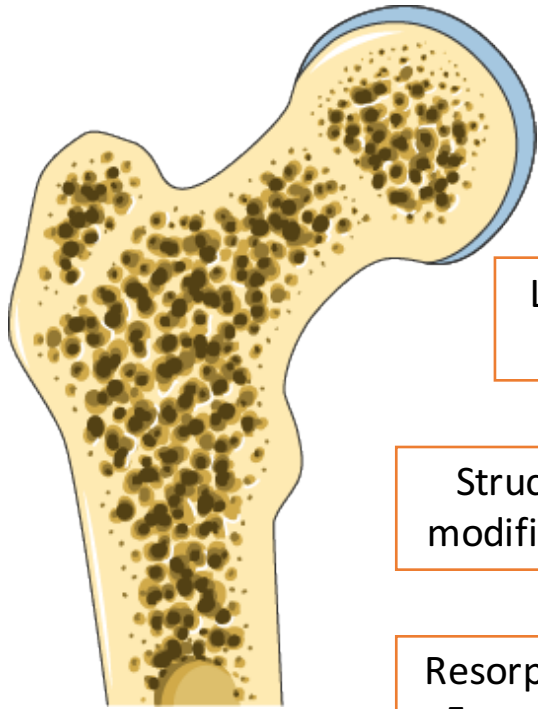
Rheumatoid Arthritis



Bone erosion

Swollen inflamed synovial membrane

Osteoporosis



Loss of bone mass

Structure modification

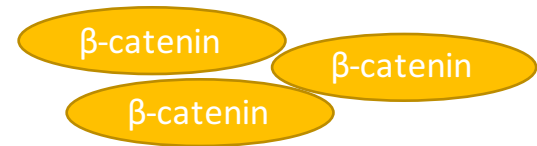
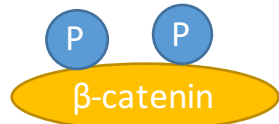
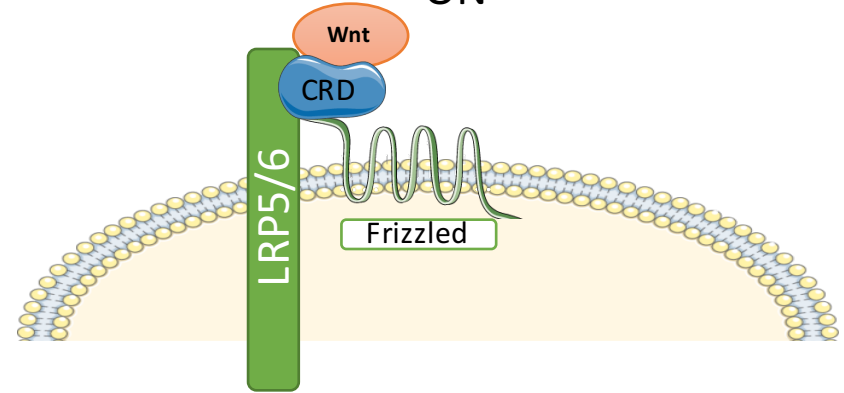
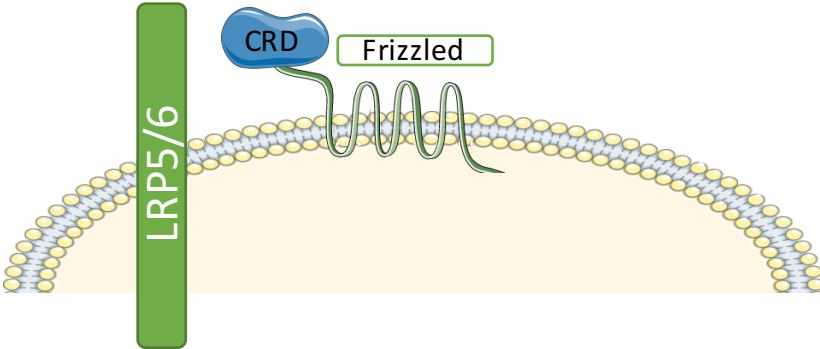
Resorption > Formation

Canonical pathway

Figure 2a

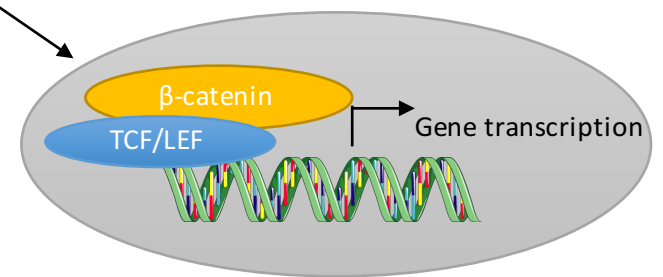
OFF

ON



Ubiquitination
Proteasome degradation

Nuclear translocation

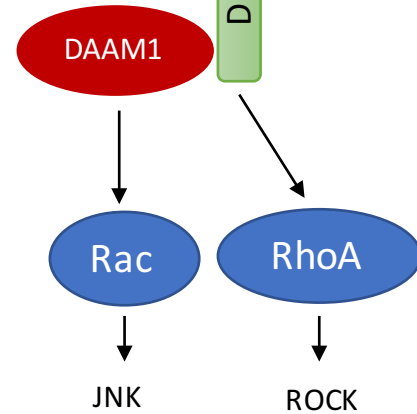
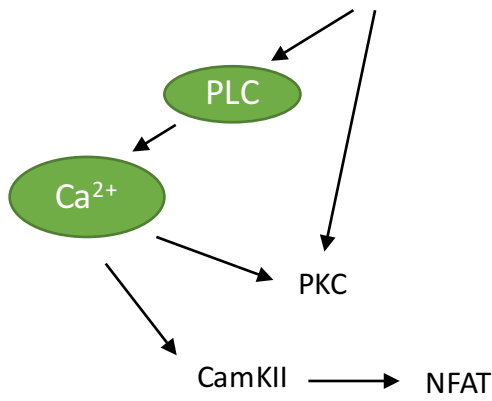
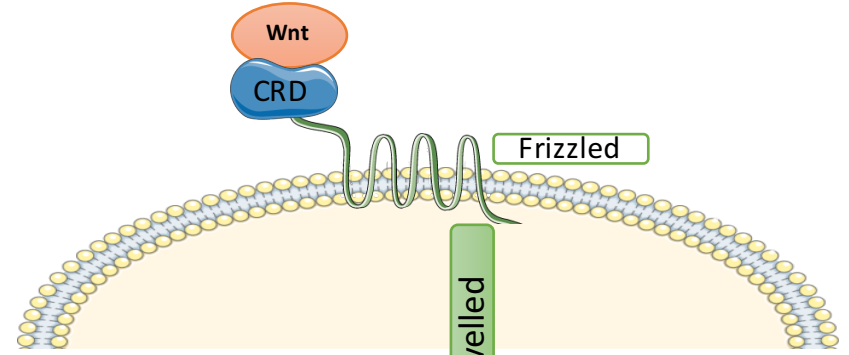
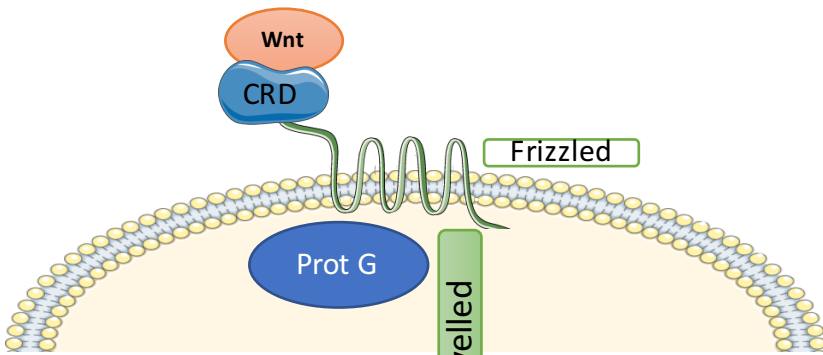


Non-canonical pathways

Figure 2b

Wnt/ Ca^{2+} pathway

PCP pathway



sFRPs

32- 40 kDa

Figure 3



- **Binding domain**²⁴
- **Heterodimerization** (CRD domain of **Frizzled receptors** and **Wnt ligands**) and **homodimerization**¹⁶

- **Heparin** binding properties²²
- Homologies with **Netrin-1**, tissue inhibitors of metalloproteinases (**TIMPs**), type 1 procollagen C-proteinase enhancer protein (**PCOLCE**), complement component proteins **C3, C4, C5**²¹
- Stimulates **osteogenesis**¹

Figure 4

WNT ON

WNT OFF

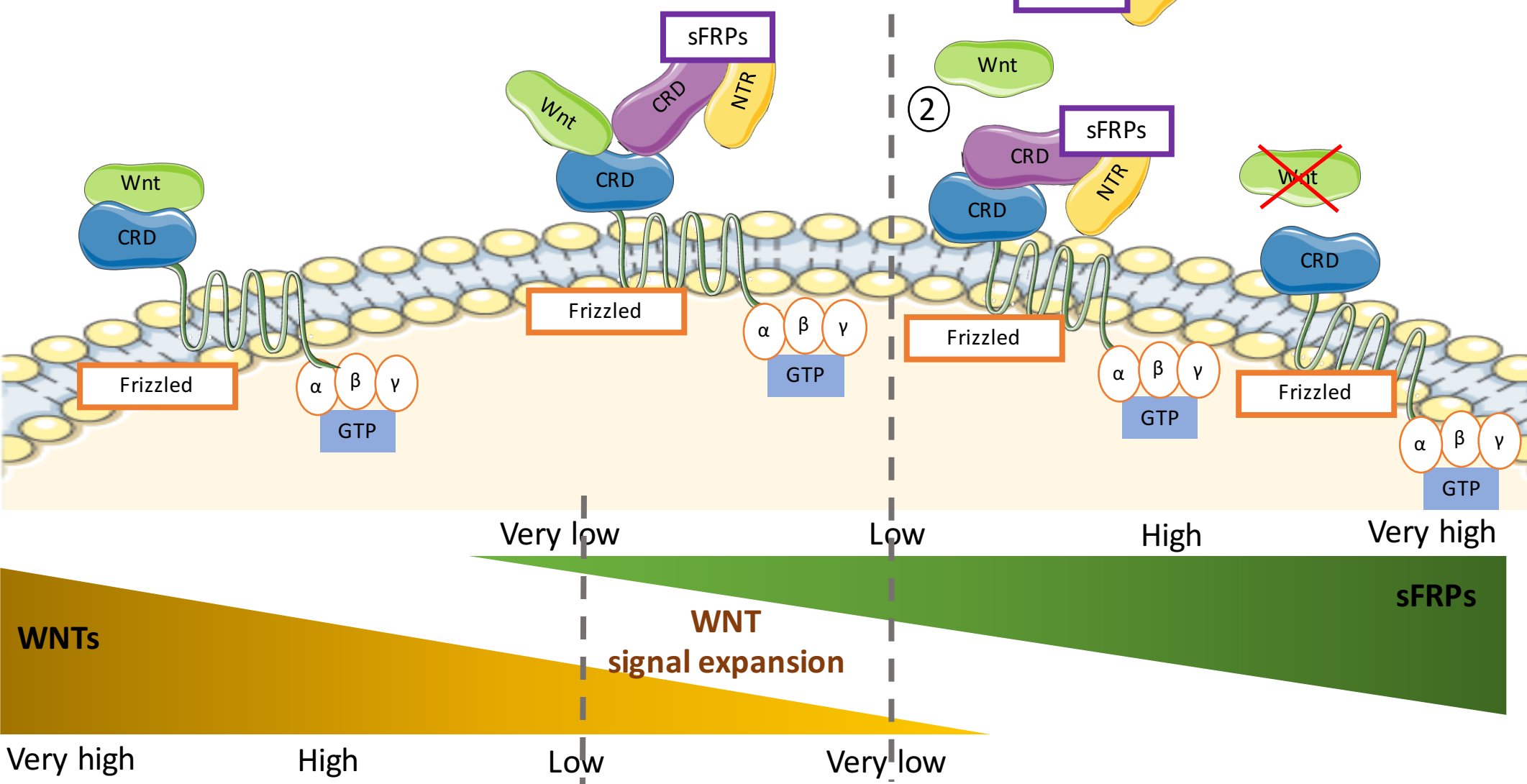


Figure 5

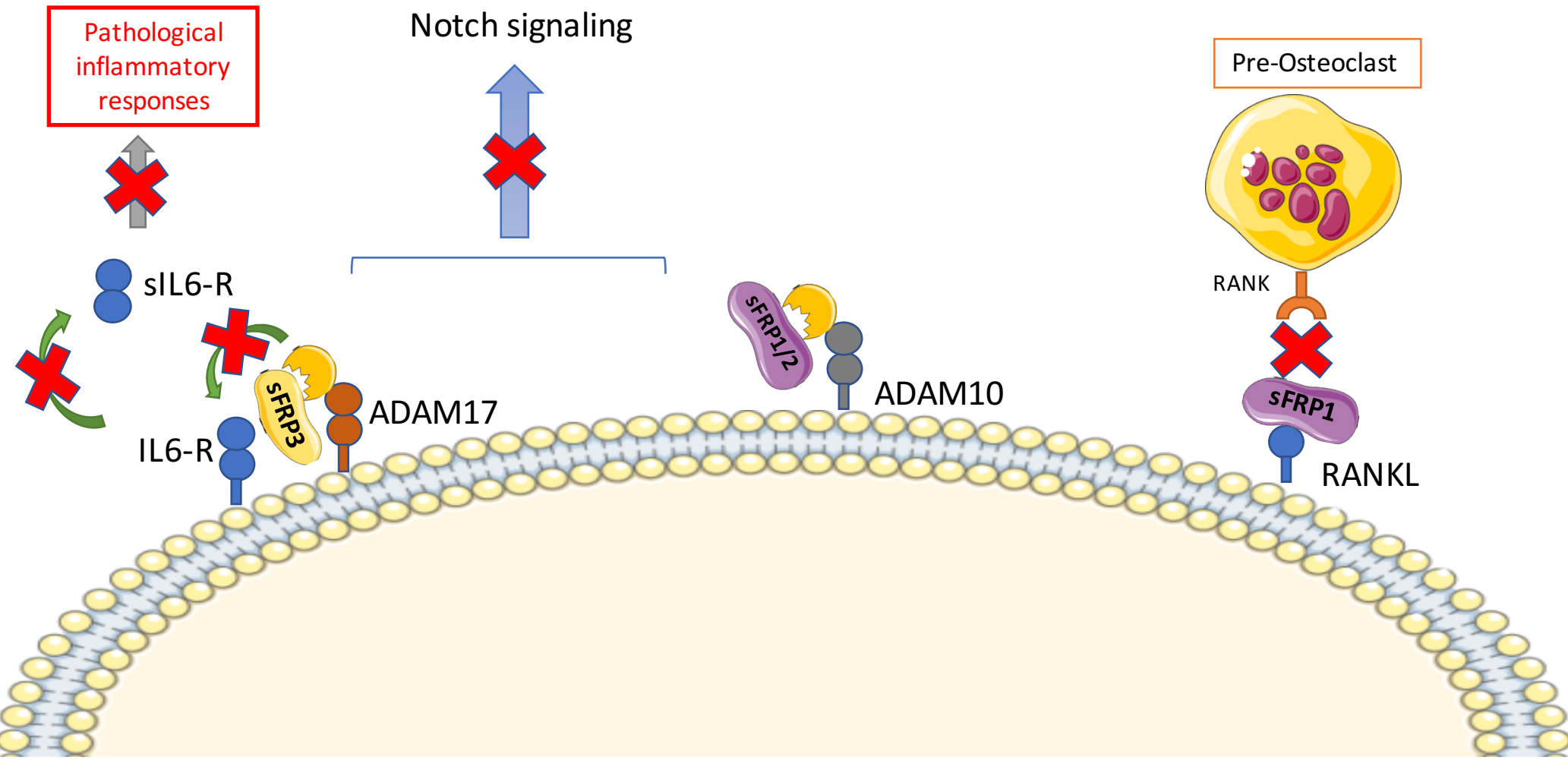


Figure 6

sFRP1 in Th17 differentiation

Wnt/ β -catenin pathway

TCF1

Repression of Th17 differentiation

↗ RORC

↗ Th17 differentiation

↗ Sensitivity of naïve CD4+ T cells to TGF- β

↗ Smad 2 phosphorylation

↗ Th17 differentiation

Figure 7

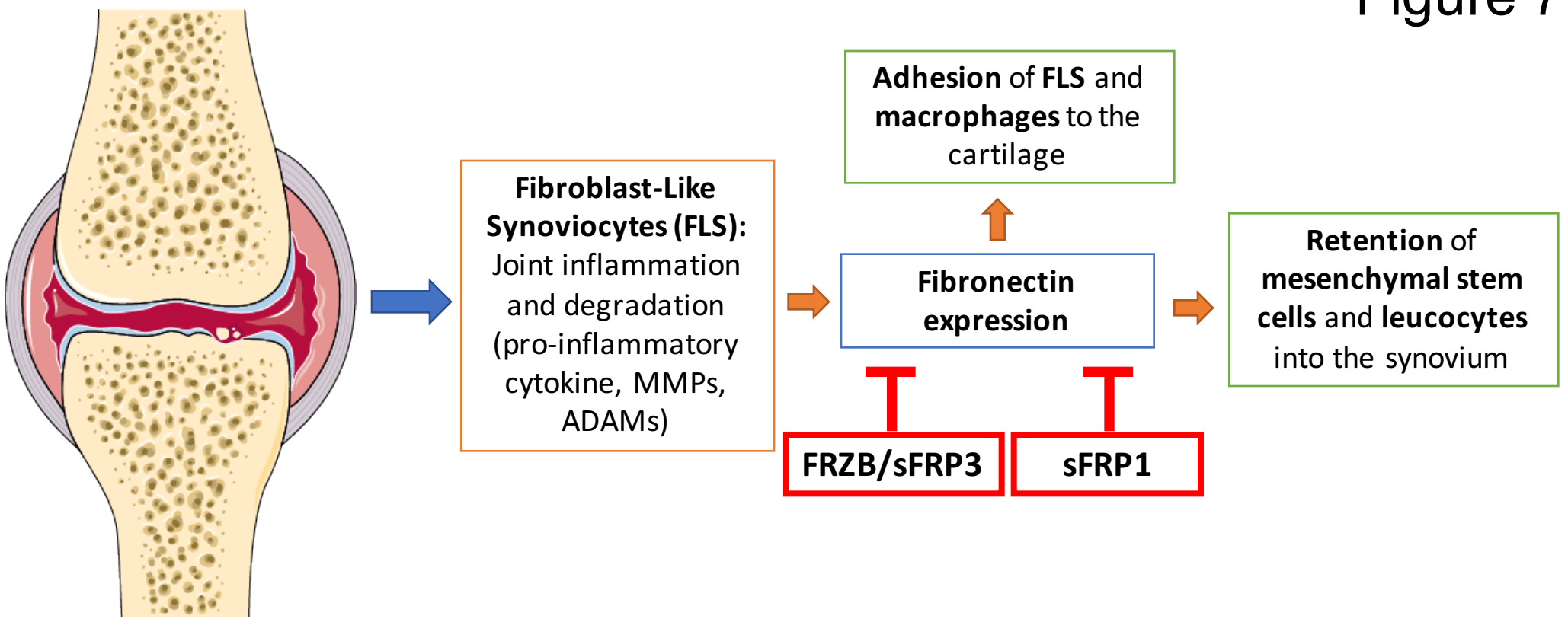
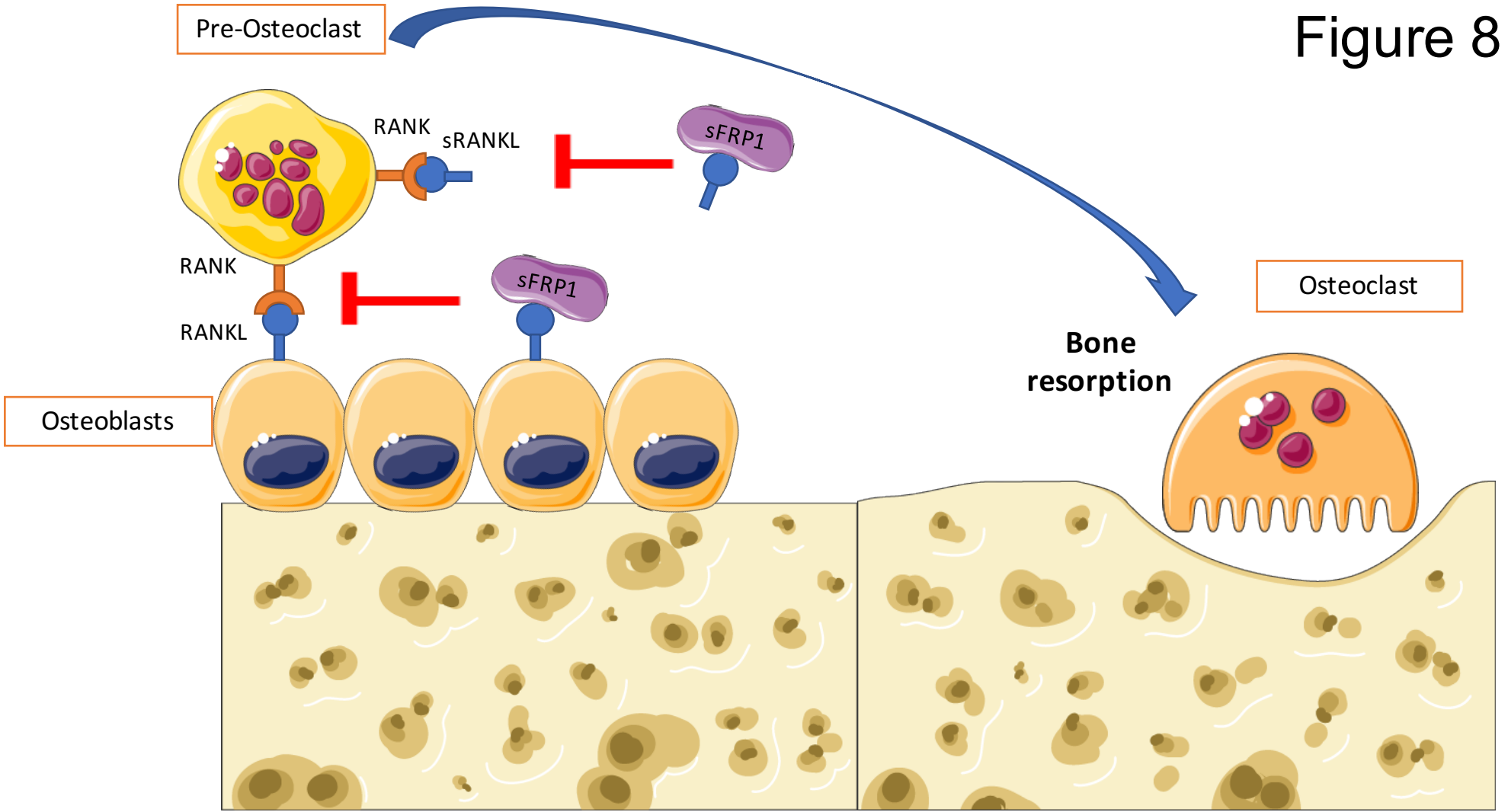


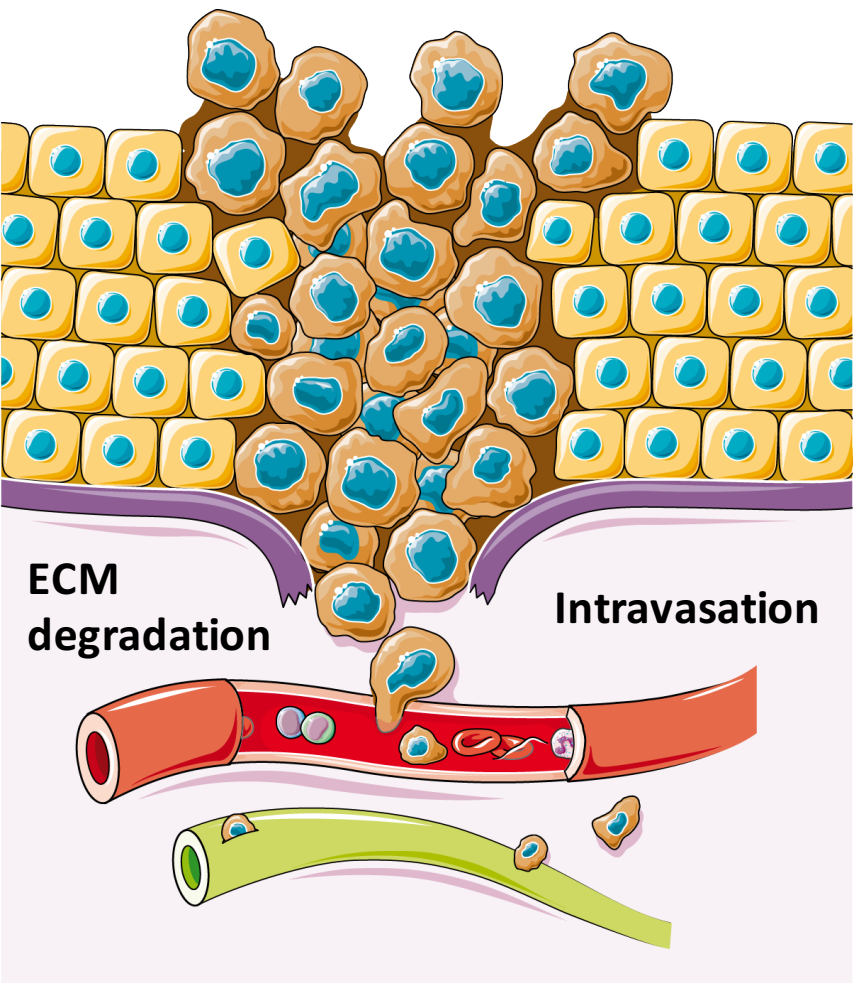
Figure 8



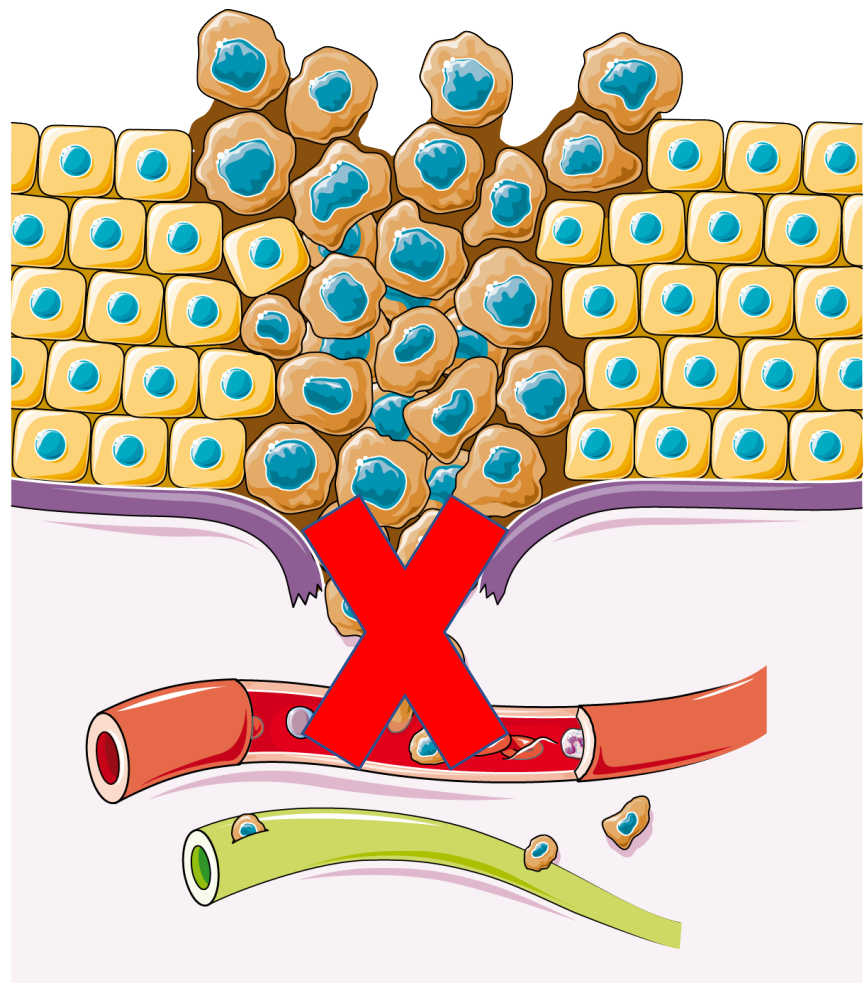
Epithelial-mesenchymal transition

Figure 9

Solid tumor



sFRP2 / sFRP4



↗ Twist, N-cadherin
↘ E-cadherin

↘ Twist, N-cadherin
↗ E-cadherin

Figure 10

