REVIEW

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An overview of molecular signaling pathways implicated in the progression of osteoarthritis

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ABSTRACT

Background. Osteoarthritis (OA) is the most prevalent joint disease worldwide, causing chronic disability in older people. Various factors are associated with its pathogenesis, including aging, obesity, joint instability, and joint inflammation.

Objectives. Since the establishment of experimental murine models with surgically induced knee joint instability many studies have revealed the major molecules or signaling pathways responsible for OA. The aim of our study is to summarize the most important molecular pathways and the growth factors that are implicated in the pathophysiology of OA.

Results. Several *in vitro* and *in vivo* studies demonstrated that neovascularization, Matrix Metalloproteinases (MMPs) secretion, sclerostin as well as TGF-β -Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs) and Notch signaling pathways play important role in chondrocyte and osteochondral unit homeostasis and in the development and progression of OA.

Conclusions. However, more *in vitro* and *in vivo* studies focusing on the investigation of interactions between the growth factors and cytokines involved into the specific molecular networks that regulate the homeostasis of articular cartilage and OA pathogenesis is deemed necessary.

KEY WORDS: Osteoarthritis, angiogenesis, Matrix Metalloproteinases, Sclerostin, Fibroblast Growth Factors, TGF-β, Bone Morphogenetic Proteins, Notch signaling.

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Introduction

Osteoarthritis (OA) is the major cause of disability in the adult population affecting more than 7% of the global population, corresponding to 500 million people worldwide. The prevalence of OA is high among the elderly (≈70%) and can lead to aggravated pain and progressive dysfunction. Although in the past it was considered as a primary disorder of articular cartilage, it is now generally considered a disease of the whole joint (panarthritis), including the calcified cartilage, subchondral cortical and trabecular bone, joint capsular tissues and the synovium [1]. It must be highlighted that the structural support and the biological cross-talk between bone and cartilage, make subchondral bone and cartilage become a closely functional unit that cannot be separated [2].

OA is characterized by abnormal neurovascularization at the osteochondral junctions, the regulatory mechanisms of which remain poorly understood. The invasion of nerves and vessels in the osteochondral unit is one of its hallmarks and is the primary reason for aggravated pain [3]. Many cytokines, including semaphorins [4], netrins [5], and growth factors, such as vascular endothelial growth factor (VEGF) and nerve growth factor [6], have been found to have significant contribution in angiogenesis process. Recently, several therapeutic strategies, such as platelet-rich plasma (PRP), hyaluronic acid (HA), and mesenchymal stem cells (MSCs), have been applied to improve OA-related symptoms, enhancing the regenerative potential of the osteochondral tissue. Additionally, molecular target therapy has demonstrated promising experimental results as it was associated with stabilization or delayed progression of the OA degeneration process and, in many cases, with restoration of the normal cartilage and subchondral bone, as observed by objective assessment methods like imaging techniques or histological and immunohistological examinations) [7 - 8]. Therefore, further *in vitro* and *in vivo* research on the molecular signaling pathways involved in the degeneration process during OA progression is deemed necessary.

Aim of the present study is to summarize the main molecular pathophysiological mechanisms that are implicated in cartilage and subchondral bone degenerative changes of OA and to unveil possible target therapeutic options that have potential restorative properties *in vivo*.

Extracellular Matrix (ECM) Degeneration and Angiogenesis

The cartilage ECM is mainly composed of collagen fibers polysaccharides, secreted enzymes and proteoglycan molecules. It serves as a protective structure for cartilage against elastic and shear loadings, but it also regulates the chondrocyte behavior via matrix–cell interactions [9]. Moreover, the collagenous proteins demonstrate crucial structural and mechanical role in the connective tissue, and in the bony tissues are mainly composed by types I, III and V. Collagen fragmentation is mediated by two distinct pathways. In the first, collagen degradation is mediated by secreted or membrane proteases. In the second, the collagen turnover occurs intracellularly through the urokinase plasminogen activator receptor-associated protein uptake (uPARAP/Endo180). After the uPARAP-induced turnover, collagen fragments are delivered to the lysosomes, where they are degraded by cathepsins B, L, N, and K under acidic conditions [10-11]. Matrix metalloproteinases (MMPs) are involved in both processes [12]. MMPs are a family of at least 24 zinc-dependent endopeptidases, capable of degrading all ECM components. In humans, the MMP family is consisted by 24 genes encoding 23 MMPs. MMP-23 is coded by two identical genes at chromosomal 1 (MMP-23A and MMP-23B). The classification of MMPs is based on a) their location in the ECM matrix (soluble) or on the cell membrane (insoluble), b) their structural appearance and substrate affinity. According to this classification they divided in six subgroups. The collagenases (MMP-1, MMP-8 and MMP-13), the gelatinases (MMP-2 and -9), the stromelisins (MMP-3, -10 and -11), the matrilysins (MMP-7 and -26), the membrane type ones (MMP-14, -15,,-16,-17 and -24) and finally the others (MMP-12, -18, -19, 20, -21, -22, -23, -27 and -28).c) their chronologically discovery. The MMPs -4, -5 and -6 are not included, because they have identical structural and functional similarities with other members of the list. Most MMPs

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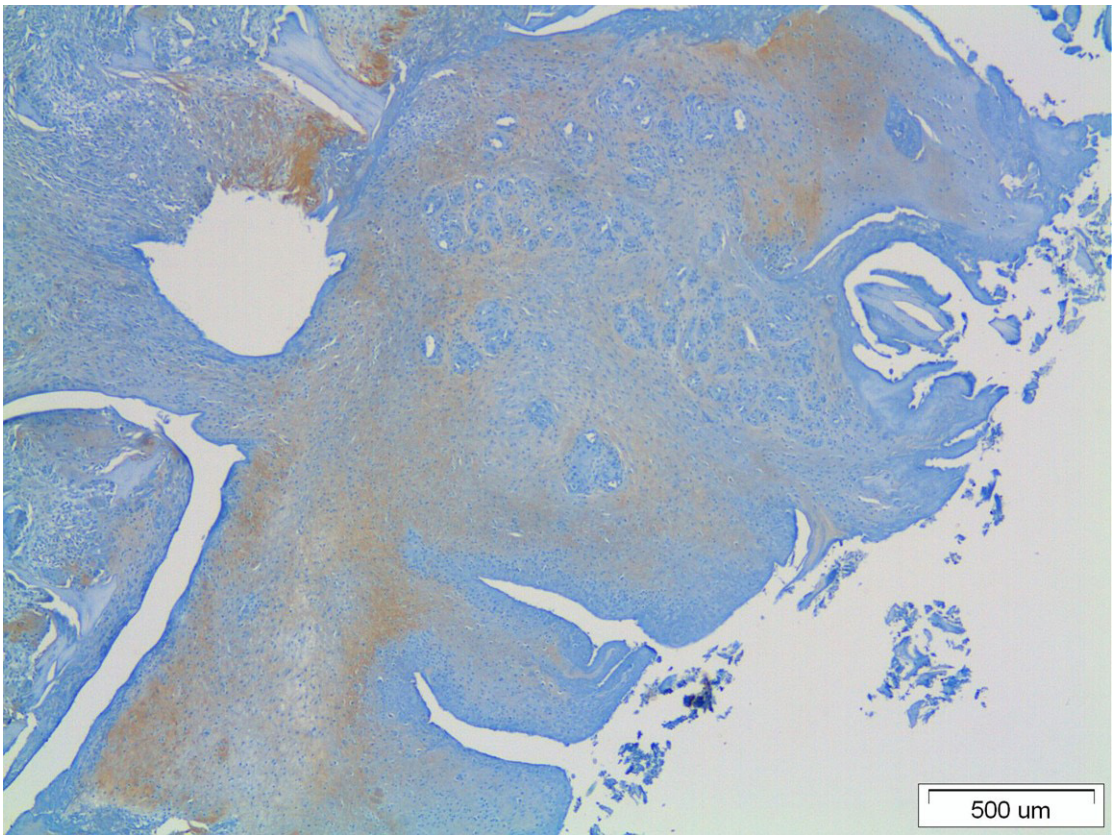

Figure 1: *Increased immunolocalization of MMP-1 in the cartilage and subchondral bone of a immunohistological section in OA patient with Mankin score 8 (Magnification 4X)*

are secreted into the extracellular space immediately after synthesis as proenzymes (pro-MMP) and are activated by proteolytic cleavage in the extracellular space. Specifically, the pro-MMPs are activated by proteolytic cleavage of the zinc-thiol interaction between the cysteine on the pre-domain and the Zn+2 on the catalytic domain by serine proteases or active MMPs, denominated as the "cysteine-switch mechanism" [13].

MMPs and a disintegrin and metalloproteinase production with thrombospondin motifs (ADAMTS) initiate ECM breakdown in OA **(Figure 1)**. Through the bone morphogenetic protein (BMP) pathway, the degradation of type II collagen (Col2A1) promotes the hypertrophy of chondrocytes, accelerating the degenerative alterations of OA [14]. Moreover, cartilage mineralization has also contributed to the OA development [15].

The activation of 2A-adrenergic receptor signaling pathway via the extracellular regulated protein kinases 1 and 2 (ERK1/2) and protein kinase A (PKA) pathways stimulate the synthesis of matrix degradation-associated enzymes, such as MMP-3 and MMP-13. Osteopontin, an inflammatory agent, also triggers the production of MMPs through the NF-κB signaling pathway [16].

The HTRA1–DDR2–MMP-13 axis is essential for ECM breakdown. This procedure begins after the increased expression of high-temperature requirement A1 (HTRA1) and the breakdown of pericellular matrix components, including type VI collagen. Col2A1 can also activate the transmembrane protein Discoidin Domain Receptor Tyrosine Kinase 2 (DDR2) in the absence of a pericellular matrix. DDR2 ultimately triggers MMP-13 leading to OA degenerative changes [17].

Healthy adult joint cartilage does not contain any blood vessels or nerves. Contrariwise, the osteoarthritic (OA) cartilage is invaded by blood vessels from the subchondral bone [18. In general,

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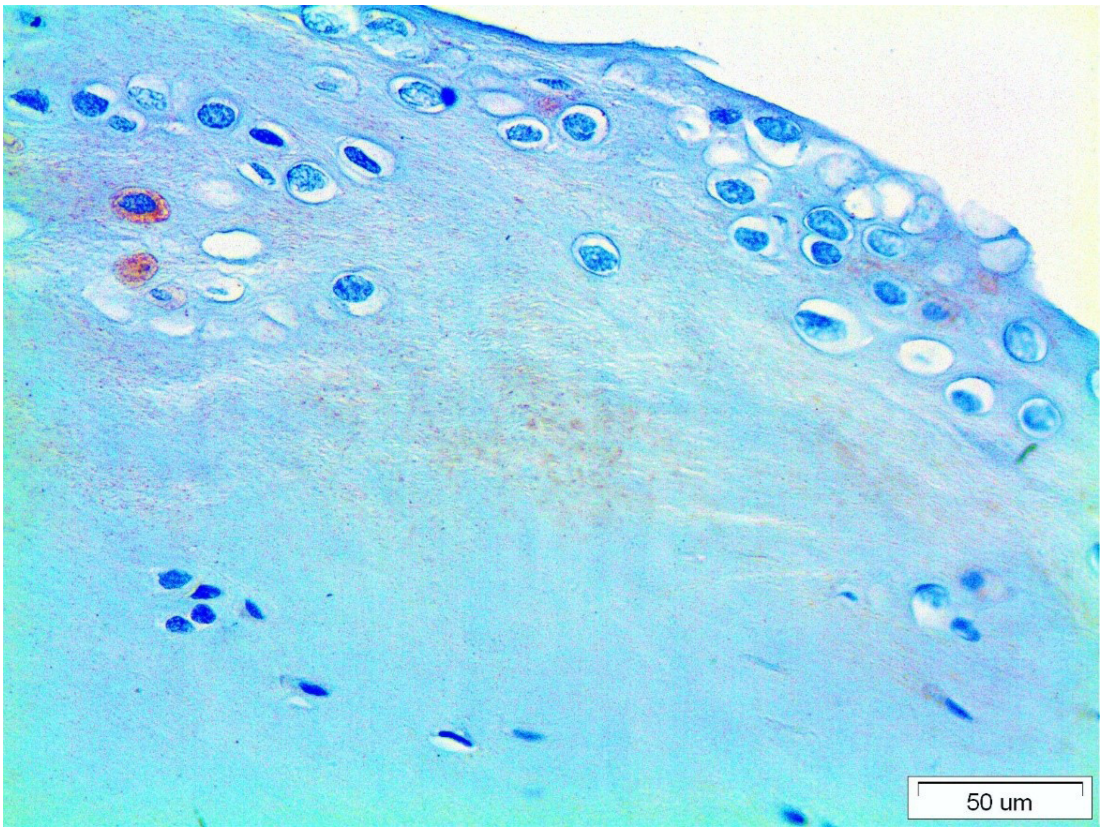

Figure 2: *Increased expression of the angiogenic growth factor Pleiotrophin (PTN) in the chondrocytes and Extracellular matrix of a immunohistological section in OA patient with Mankin score 7 (Magnification 40X)*

cartilage angiogenesis results from the imbalance between pro- and anti-angiogenic factors. The imbalance leads to increased production of pro-angiogenic factors and/or decreased production of anti-angiogenic factors [19]. VEGF-A, the best studied growth factor, is associated with MMP production, Additionally, inflammatory mediators induce angiogenesis and trigger MMP production. For example, TNF-α may contribute to the regulation of the expression of MMP-9 and MMP-14 that are crucial for vessel progression into the ECM [20. Many research studies have detected upregulation of MMPs in the osteoarthritic serum and joint tissues. Specifically, MMP-1 **(Figure 1)** and MMP-12 expression were increased in the osteochondral unit in patients with osteoarthritis (OA) and there was a positive correlation between their expression and OA severity [21,22] Furthermore, immunodetection of the collagenases (MMPs 1, 8, and 13) and stromelysin 1 (MMP-3) was demonstrated in a proportion of chondrocytes of human specimens that had OA-related degenerative matrix changes [23]. MMP-13, the collagenase with the strongest activity against type II collagen, seems to have a key-role in OA-associated joint destruction [335], while MMP-3 has been evaluated as a prognostic tool in prediction of the disease progression. Patients with increased plasma MMP-3 have increased possibility for OA progression over a 30-month period. Paradoxically, MMPs can decrease angiogenesis by cleaving the receptor binding sites of pro-angiogenic factors.

Additionally, several transcription factors, including hypoxia-inducible factor-1 (HIF-1), promote VEGF expression [24]. The protein dickkopf-related protein-1 (DKK-1) was detected in high concentrations in the synovial fluid of OA patients. It was suggested that DKK-1 and high-mobility group box 1 (HMGB1) led HIF-1 nuclear localization, and increase the expression of VEGF [24-25]. Inflammatory cytokines such as Interleukin-6 (IL-6) and IL-

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1, also, stimulate the expression of VEGF through the elevation of VEGF transcription in the nucleus. ERK1/2 stimulates the estrogen-related receptor γ (ERR γ). IL-1 which is a direct stimulus for NF-κB production [26].

VEGF belongs to a family that includes at least six members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placental Growth Factor (PIGF). VEGF-A or VEGF, which is the most abundant form, is playing a key-role in proliferation, migration and activation of endothelial cells and is a potent inducer of vascular leakiness. VEGF-B is important for new vessel development during embryogenesis] while VEGF-C and VEGF-D are involved in lymphangiogenesis. VEGF binds to the extracellular domains of two tyrosine kinase receptors, VEGF receptor I and VEGF receptor II . VEGF receptors also include VEGF receptor III, Neuropilin 1 (Npr1) and Neuropilin 2 (Npr2) [39]. VEGFR-II is the main VEGF signaling receptor and is considered the responsible receptor for VEGF-induced regulation of angiogenesis, vasculogenesis and vessels permeability [32]. VEGF is generated by articular cartilage chondrocytes and modulates autocrine levels of MMP-13 and tissue inhibitor of metalloproteinase-1 (TIMP-1). The reduction of TIMP expression and the overexpression MMPs disrupt the circulation of ECM components, collagen, and proteoglycans. VEGF may increase articular cartilage deterioration by activating osteoclasts and allowing blood vessels to penetrate the cartilage [27]. Moreover, VEGF appears to be involved in OA specific clinical pathologies including cartilage degeneration, osteophyte formation, subchondral bone cysts and sclerosis, synovitis, and pain. Moreover, a wide range of studies suggested that inhibition of VEGF signaling reduces OA progression.

The breakdown of hyaluronic acid (HA) and the increased activity of free radicals are correlated with reduced joint hydration. When the intra-joint pressure begins to overpass the capillary pressure, the accompanied transient hypoxia can result in joint degeneration defects. Reoxygenation is observed when the stress on the joint is decreased, and joint degeneration changes are halted.

Hypoxia and reperfusion cycles are closely related with the production of free radicals. Free radicals accelerate HA breakdown, lowering synovial fluid viscosity and increasing friction between joint surfaces [28]

Pleiotrophin (PTN) is a secreted growth factor with molecular weight of 18kDa which appears to have an important role in angiogenesis [29]. NMR studies have shown that PTN structure is consisted by two β-sheet domains containing three antiparallel β-strands, homologous to the thrombospondin type 1 repeat (TSR-1) [30] Thrombospondin is involved in bone development and remodeling, supporting the notion that PTN is a significant regulator in these biological processes [31] PTN binding to its receptors leads to its biological actions. N-syndecan, which contains, heparan sulfate chains, was the first PTN receptor to be detected [32]. N-syndecan is expressed by pre-osteoblastic cells and is implicated in the regulation of osteoblast recruitment [33]. The best studied PTN receptor is receptor protein tyrosine phosphatase beta/zeta (RPTPβ/ζ), which contains of a large glycosylated extracellular domain, a transmembrane region and a cytoplasmic portion that contains two repeated tyrosine phosphatase domains, which have a characterized role in PTN-induced cell migration. Another important PTN receptor that regulates the stimulatory or the inhibitory effect of PTN on cell migration is αvβ3 integrin, which forms a functional complex with RPTPβ/ζ on the cell surface of endothelial cells [33] Finally, anaplastic lymphoma kinase (ALK), a 220kDa receptor tyrosine kinase, has been also identified as PTN receptor, but current research studies demonstrated that ALK is not directly activated by PTN, but through PTN-dependent inactivation of RPTPβ/ζ. *In vitro* studies displayed that recombinant human PTN had chemotactic effects on both human osteoblastic and endothelial cells [350] whereas immunolocalization of PTN on both osteoblasts and endothelial cells was observed in the newly formed woven bone [34] Moreover, PTN induces hypertrophy during chondrogenic differentiation of human bone marrow stem cells, increasing the transcription of hypertrophic chondrocyte markers, such as MMP13, collagen 10 and alkaline phosphatase, and resulting in enhanced calcification and higher con-

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tent of collagen 10 [35]. PTN is also over-expressed in degenerative diseases like osteoarthritis, where increased PTN levels were identified in patient's synovial fluid [36] and serum, as well as in chondrocytes and subchondral bone osteocytes [36].

TGF-β and BMP Signaling

BMPs are members of the TGF-β (Transforming Growth Factor-β) family, a large growth factor family. More than thirty members of the BMP family have been identified and are categorized into several subgroups. Individually, the members of the BMP subfamily are called either BMPs, Osteogenic Proteins (OP-1, OP-2, OP-3 for BMP-7, BMP-8 BMP-8b respectively), Growth Differentiation Factors (GDF-1, GDF-2/BMP-9, GDF-3, GDF-5/BMP-14, GDF-6/BMP-13, GDF-7/BMP-12, GDF-8 to GDF-10 and GDF-11/BMP-11), or Cartilage-Derived Morphogenetic Proteins (CDMP-1/BMP-14 and CDMP-2/BMP-13) [216-225]. Based on their amino acid homology and structural similarities, BMPs are classified into several subclasses. Members of the first subclass are BMP-2 and -4, which have 80% amino acid homology and differ in the amino terminal region. BMP-2 contains an extra heparin-binding domain. The BMPs that constitute the second subclass are larger proteins having 78% amino acid similarity between the subgroups and involve BMP-5, -6 and -7. The BMPs of the third subclass are distantly related to the above molecules [37]. BMPs are synthesized intracellularly in a large inactive precursor form of about 50 – 100 amino acids that contains an amino-terminal signal peptide, a predomain and a mature peptide. After the signal peptide cleavage, the precursor polypeptide undergoes glycosylation and dimerization. Once secreted, the predomain is cleaved, allowing the mature and active BMP proteins to act as a dimeric biomolecule. Mature BMP is considered the BMP derived after the proteolytic cleavage of the carboxyl-terminal region and is secreted mainly as homodimer or as heterodimer [38].

Two different types of membrane serine/threonine kinases receptors for BMPs have been recognized: type I (BMPR-I) and type II (BMPR-II) [39]. Once BMP binds to BMPR-I, a ligand/receptor complex is formed, which connects with the BMPR-II receptor. This interaction in turn phosphorylates the cytoplasmic glycine/serine domain (GS domain) of BMPR-I, stimulating the BMP signaling cascade [40]. BMPs also, play remarkable role in bone metabolism and several studies have demonstrated the role of several antagonists and regulators of the BMP-induced pathways. The activities of BMPs are critical for a series of molecular events that result in angiogenesis, chondogenesis and osteogenesis [41]. The BMP-induced molecular signaling pathways can be regulated in three stages: through the extracellular signals, at the membrane or receptor level and through intracellular proteins and pathways.

The TGF-β signal pathway initiates intracellular signaling following the creation and activation of a heteromeric complex of types II and I serine/threonine kinase receptors, followed by the phosphorylation of particular Smad proteins, R-Smads. The phosphorylated R-Smads can heterodimerize with co-Smad, Smad4, ultimately translocating to the nucleus and activating the transcription of target genes [42].

In the TGF-β/Smad3 signaling pathway, sphingosine 1-phosphate (S1P), a bioactive lipid, is produced to function as an intracellular mediator or extracellular ligand for different receptors, resulting in inflammation, cell migration, and angiogenesis. The interaction between TGF-β/Smad3 and S1P/S1P3 and Smad3/S1P3 signaling in chondrocytes may play a role in the development of OA [43]. Additionally, it has been documented that overexpressing TGF-β1 causes aberrant subchondral bone remodeling that causes mice to develop OA and degrade articular cartilage [44]. Indeed, a recent study reported that the inhibition of TGF-β signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis [45]

Fibroblast Growth Factors (FGFs)

Along with the VEGF family, the FGF family of growth factors is one of the two best studied growth factor families in angiogenesis. The mammalian FGF family contains 22 members so far [46]. The FGF growth factors, except for FGF11-14 (which are intracellular) and FGF-21 and -23 (which act in an endocrine manner), facilitate their biological activ-

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ity by binding to the 4 transmembrane FGF Receptors (FGFR1-4) [47]. From the 4 FGFRs, FGFR1-3 are expressed in osteoblasts [46] and we have identified a similar expression pattern in endothelial cells (no detectable expression of FGFR4 in Human Umbilical Vein Endothelial Cells (HUVECs) leading to the hypothesis that the FGFR1-3 are mostly important for their biological activity in bone regeneration and angiogenesis. This is confirmed with data showing that mutations to FGFR1-3 are directly related with bone phenotypes, such as dwarfism and achondroplasia [47]. Among the 22 FGF ligands, FGF-2, FGF-4, FGF-7, FGF-8, FGF-9, FGF-10, FGF-17 and FGF-18 are expressed in the developing skeleton [48].

The most solid evidence on the role of the FGF family in angiogenesis has derived from skeletal phenotypes of mice deficient for FGF-2, -8, -9, -10, -18 and -23 [49], which clearly demonstrates the importance of each one of these growth factors on bone development and remodeling. Below we will mention key characteristics and properties of the ones that play significant role for the bone healing and regeneration process. FGF signaling may be increased in OA, as evidenced by the finding that ablation of FGRF1 in chondrocytes being associated with reduced OA development in particular OA models [50]. In particular, inactivated FGFR1 signaling ameliorates OA progression partially by promoting autophagic activity [50].

Notch Signaling

The recent identification and characterization of tip cell function during vessel sprouting in the retina [51] and the identification of the Notch signaling as the responsible mechanism for tip cell versus stalk cell physiology in the same model [52] have elucidated one pivotal signaling mechanism for angiogenesis. In every vessel sprouting process there is one endothelial cell invading, which is morphologically distinct, as it has extensive filopodia. The invading tip cell expresses high levels of the Notch ligand Delta-like 4 (Dll4) which activates Notch in the neighboring non-invading stalk cells. Activation of the Notch receptor leads to VEGFR2 downregulation and VEGFR1 upregulation impairing the response to VEGF signaling, and thus the tip cell behavior (no migration towards VEGF gradient levels) and promoting a stalk phenotype in these cells. Stalk cells proliferate more, produce less filopodia, migrate less and they are supposed to eventually form the lumen of the new vessel. It is considered that Notch expression defines the cell fate, since Notch presence will lead to a stalk cell behavior, whereas its absence is related to increased sprouting. Indeed, abnormal sprouting leads to a very dense vessel network, however poorly functional due to limited perfusion [53]

The Notch signaling system regulates the molecules involved in cartilage production and breakdown and hence plays a dual function in cartilage maintenance [54]. It is well known that Notch signaling is critical in the angiogenesis of condylar cartilage and disc, which is required to form OA [54]. Recent research has shown that changing Notch signaling may cause OA. Specifically, Notch 1 and 2 receptors are highly expressed in articular chondrocytes and are localized at the cell surface in normal mouse and human articular cartilage but are translocated into the nucleus in degenerated cartilage [54]. Inhibition of Notch signalling by Rbpj knockout in chondrocytes after skeletal development suppresses OA development in a murine surgical model, and injection of the γ-secretase inhibitor DAPT into the knee joints of the wild-type OA model mice results in a similar protective effect [54]. Mmp13 expression is increased by overexpression of Notch-ICD in mouse primary chondrocytes and chondrocyte cell lines and is decreased in Rbpj-knockout cartilage.

Sclerostin

Sclerostin is a soluble antagonist of canonical Wnt signaling and a strong inhibitor of bone formation, almost exclusively secreted by osteocytes [55]. It belongs to the cystine knot family and is a product of the sclerostin (SOST) gene [56]. Secreted Wnt inhibitors, such as sclerostin, are a group of proteins which facilitate their activity, binding extracellularly to the co-receptor LRP5/6 [55]. Sclerostin is a glycoprotein with a molecular weight of 24 kDa and is secreted almost exclusively by osteocytes and to a lesser extent by other cell types like osteoclasts, renal and vascular cells [57]. Sclerostin substantially regulates bone min-

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eralization processes and is a potent anti-anabolic agent in the skeleton. Osteocytes reduce the release of sclerostin after mechanical stimulation [57]. Reduced in vivo activity of sclerostin leads to increased bone mass and strength [58], while increased expression of sclerostin in experimental models is associated with reduced bone mass [59]. Moreover, many research studies reported that SOST is upregulated in OA acting as a rescue mechanism to prevent further degenerative changes of the joint. It antagonises inflammation-induced cartilage catabolism while it preserves chondrocyte anabolic activities. It also prevents abnormal bone mineralisation and osteophyte formation [60]. Specifically, chondrocyte SOST immunoexpression was remarkably elevated in the focal area of cartilage damage in surgically induced OA in sheep and mice models as well as end-stage in human OA. Contrariwise, the expression of SOST was decreased in the osteocytes of subchondral bone in sheep OA in association with bone sclerosis. SOST was biologically active in chondrocytes, inhibiting Wnt-β-catenin signaling and catabolic metalloproteinases MMPs and distintegrin or metalloproteinase with thrombospondin repeats (ADAMTS)] expression, but also decreasing the mRNA levels of aggrecan, collagen II and tissue inhibitors of metalloproteinases (TIMPs). Despite this mixed effect, SOST dose-dependently inhibited IL-1α-stimulated cartilage aggrecanolysis *in vitro* [60].

Conclusion

Recent clinical and experimental evidence demonstrated that many signaling pathways regulate the homeostasis of articular chondrocytes and OA development activating many growth factors and cytokines. Comprehensive understanding of the molecular networks including articular chondrocyte proliferation differentiation and the implicated molecules is needed for further elucidation of OA pathogenesis and progression.

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