Review Article

Ser/Thr-phosphoprotein phosphatases in chondrogenesis: Neglected components of a two-player game

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Abstract

Protein phosphorylation plays a determining role in the regulation of chondrogenesis *in vitro*. While signalling pathways governed by protein kinases including PKA, PKC, and mitogen-activated protein kinases (MAPK) have been mapped in great details, published data relating to the specific role of phosphoprotein phosphatases (PPs) in differentiating chondroprogenitor cells or in mature chondrocytes is relatively sparse. This review discusses the known functions of Ser/Thr-specific PPs in the molecular signalling pathways of chondrogenesis. PPs are clearly equally important as protein kinases to counterbalance the effect of reversible protein phosphorylation. Of the main Ser/Thr PPs, some of the functions of PP1, PP2A and PP2B have been characterised in the context of chondrogenesis. While PP1 and PP2A appear to negatively regulate chondrogenic differentiation and maintenance of chondrocyte phenotype, calcineurin is an important stimulatory mediator during chondrogenesis but becomes inhibitory in mature chondrocytes. Furthermore, PPs are implicated to be important mediators during the pathogenesis of osteoarthritis that makes them potential therapeutic targets to be exploited in the close future. Among the many yet unexplored targets of PPs, modulation of plasma membrane ion channel function and participation in mechanotransduction pathways are emerging novel aspects of signalling during chondrogenesis that should be further elucidated. Besides the regulation of cellular ion homeostasis, other potentially important novel roles for PPs during the regulation of *in vitro* chondrogenesis are discussed.

Keywords: osteoarthritis; cartilage; protein kinase; calcium signalling; ion channel; calcineurin; chondrocyte differentiation

Abbreviations:

ADAMTS, "A Disintegrin And Metalloproteinase with Thrombospondin Motifs"; BMP, bone morphogenic protein; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaN, calcineurin; CNA, CNB, calcineurin catalytic (A) and regulatory (B) subunits; COX, cyclooxygenase; CREB, cAMP response element binding protein; CsA, cyclosporine A; ECM, extracellular matrix; ERK1/2, extracellular signal-related kinase 1/2; FGF; fibroblast growth factor; GADD34, growth arrest and DNA damage protein-34; HDAC, histone deacetylase; IP_3R, inositol trisphosphate receptor; MAPK, mitogen-activated protein kinase; MEF, myocyte enhancer factor; MMP, matrix metalloproteinase; NFAT, nuclear factor of activated T lymphocytes; NF-κB, nuclear factor-kappa B; NO, nitrogen oxide; OA, osteoarthritis; OKA, okadaic acid; PACAP, pituitary adenylate cyclase activating polypeptide; PK, protein kinase; PKA, PKC, protein kinase-A, protein kinase C; PP1, PP2A, PP2B, protein phosphatase 1, 2A, 2B; PP, phosphoprotein phosphatase; PTHrP, parathyroid hormone related peptide; ROS, reactive oxygen species; RyR, ryanodine receptor; SAPK/JNK, stress-activated protein kinase/c-jun N-terminal kinase; SOCE, store-operated Ca^{2+} entry; SSC, synovial stromal cell; TGF-β, transforming growth factor beta; TNF, tumour necrosis factor; TRPV, transient receptor potential vanilloid; TβR, TGF-β receptor

1. Introduction

The formation and maturation of tissues that comprise the musculoskeletal system in the developing embryo is a truly enigmatic process. Formed as early as week 4 in the human embryo, the undifferentiated embryonic connective tissue (mesenchyme) that eventually gives rise to the skeleton develops primarily from components of mesoderm [1]. In addition, cartilaginous parts of the cranium are derivatives of ectomesenchyme of neural crest origin. It is from this mesenchyme that the histogenesis of cartilage commences at condensed sites called chondrification centres that appear starting from week 5; mesenchymal cells committed towards the osteochondrogenic lineage differentiate into chondroblasts and start to secrete the specialised components of the cartilage extracellular matrix (ECM).

The transition from elongated mesenchymal cells to ovoid chondroblasts is a highly complex procedure that involves changes in gene expression [2], activation and/or inhibition of various signalling pathways [3, 4], alterations in cytosolic Ca^{2+} concentration [5, 6], and differentiation stage-dependent expression and function of plasma membrane ion channels [7, 8]. Because of the complexity of programmed alterations necessary for chondrogenic differentiation, the details of this process are still incompletely understood. Besides elucidating the molecular mechanism that drives chondrogenesis, cartilage research is also aimed at providing a better understanding of pathological processes that affect the normal functions of this tissue, causing debilitating disorders. Osteoarthritis (OA) is the most prevalent degenerative joint disease and source of chronic pain for millions of people worldwide. Causes of OA are complex with interplay between mechanical, genetic and lifestyle factors [9]. At the late stages of the disease, OA is characterised by degradation of articular cartilage, synovial inflammation, and osteophyte formation [10]. At present, there are no effective disease modifying drugs available that are specifically targeted to OA; therefore, there is a pressing need to identify and develop novel therapeutic agents to halt or

even reverse disease progression [11]. Lack of effective and specific drugs against OA are at least partially a consequence of the fact that the molecular nature of the disease is not adequately known.

This review article focuses on the role of protein phosphorylation, the most widespread mechanism for posttranslational protein modification to modulate cellular behaviour, with special emphasis on current knowledge regarding the involvement of phosphoprotein phosphatases (PPs) in the process of chondrogenesis and the pathogenesis of OA.

2. Reversible protein phosphorylation is a key regulator of chondrogenesis

Reversible posttranslational modification of proteins provides an extremely efficient means of rapid regulation of protein activity without the immediate need for *de novo* protein synthesis or targeted protein degradation. Activity of a vast number of cellular proteins is regulated by phosphorylation at select Tyr and/or Ser/Thr residues by Tyr and/or Ser/Thr-specific protein kinases (PK). In particular, approximately one-third of all proteins in a given cell have been shown to be phosphorylated at a given time; the complement of phospho-proteins in a cell is referred to as the phosphoproteome [12]. In a study that mapped phosphorylation sites in HeLa cells, phospho-Tyr sites were the least abundant (1.8%), followed by phospho-Thr sites (11.8%), and Ser residues proved to be the most frequently phosphorylated sites (86.4%) [13].

A wide array of cellular processes and functions ranging from metabolism, cellular homeostasis, migration, cell division and proliferation, differentiation, and even pathological processes such as tumourigenesis are controlled by reversible protein phosphorylation [14]. The actual level of phosphorylated cellular proteins is determined by a balance between PKs and PPs. To carry out the immense amount of modifications, approximately 500 PKs have been identified in the human genome, of which ~90 were specific for Tyr residues, and the

remaining 428 kinases preferentially phosphorylate Ser/Thr residues [15], which correlates well with the relative abundance of the phospho-Ser/Thr sites described above. Given the predominance of phosphorylation and dephosphorylation at Ser/Thr residues, and the relatively understudied role of Tyr-specific phosphatases in cartilage formation, this review article focuses on Ser/Thr-specific PPs in chondrogenesis.

Since attaching a phosphate group released from the hydrolysis of ATP to select Ser/Thr residues of protein substrates is a fairly simple process, the structural biochemistry of the PK superfamily is likewise simple; they all share a basic structure and operate with similar mechanisms [16]. At the same time, PKs can regulate diverse biological pathways; this is achieved by various activation mechanisms and their multiple downstream signalling targets. Many Ser/Thr-specific PKs have been reported over the previous decades that proved to be essential regulators of the formation of chondroblasts and then chondrocytes. In the following section we provide a brief overview of main Ser/Thr-specific PK families reported to control various steps of chondrogenesis.

2.1. Ser/Thr-specific PKs regulate chondrogenesis

Given that chondrogenesis is a highly complex process, it is not surprising that all main PK families have been reported to play a fundamental role in its molecular regulation. Cyclic AMP-dependent protein kinase (PKA) activity detectable in cell homogenates of developing limb buds of early chicken embryos [17] was found to enhance *in vitro* chondrogenesis and to increase phosphorylation of the cAMP response element binding protein (CREB) [18]. Unlike PKA, the collection of enzymes that constitutes the protein kinase C (PKC) subfamily is traditionally subdivided into classic, novel and atypical PKCs. PKC activity has also been detected in chondrogenic micromass cultures [19], and a wide array of functions has been associated with PKC isoenzymes since then. These include, but are not limited to, actin

cytoskeleton remodelling, cell adhesion molecules, ion channel activity, and modulation of other signalling pathways (reviewed in [4]). PKA and PKC activities have been proved to be closely linked; PKA, in particular, regulates chondrogenesis by modulating N-cadherin expression *via* activating PKCα at the early stages of chondrogenesis [20].

The three major mitogen-activated protein kinase (MAPK) cascades, *i.e*. the extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-jun N-terminal kinase (JNK) pathways, are quintessential regulators of chondrogenesis (for an excellent recent review, see [3]). Consisting of a cascade of protein kinases, Raf, MAPK/ERK kinase (MEK) and ERK1/2, the ERK pathway mediates the effects of growth factors and cytokines through a plethora of downstream signalling pathways during chondrogenesis. While p38 clearly stimulates chondrogenesis in all experimental models studied [21, 22], the ERK1/2 pathway has been reported to be both negative [23, 24] and positive regulator [25, 26] of chondrogenic differentiation, depending on the species, the differentiation stage, the embryonic origin of chondroprogenitor cells, and probably the context of other concurrently active/blocked signalling mechanisms [3]. Likewise, both chondrogenesis-promoting [27] and inhibitory [28] effects for the JNK pathway have been established.

The family of Ca^{2+}/calmodulin-dependent protein kinases (CaMK) consists of 4 multifunctional Ser/Thr PKs; CaMKII (also known as eEF2-kinase) and the CaMK cascade in which CaMK kinase (CaMKK) phosphorylates and activates CaMKI and CaMKIV [29]. This pathway is of particular importance since a plethora of messages encoded by specific alterations in the amplitude, frequency and special distribution of cytosolic Ca^{2+} concentration is deciphered by these Ca^{2+}-calmodulin dependent kinases [30]. CaMKs have been implicated to mediate the effects of mechanical forces in chondrocytes [31] and are also involved in modulating hypertrophic gene expression (*e.g*. *Runx2*, *Col10a1*) during terminal differentiation of chondrocytes [32]. *Table 1* summarises the main PK families with a proven

role in chondrogenesis or mature chondrocytes.

3. Ser/Thr-specific phosphoprotein phosphatases (PPs)

For protein phosphorylation to be reversible and adjustable to the actual needs of the organism and/or cell, the actions of both phosphorylation (*i.e.* PKs) and dephosphorylation (*i.e*. PPs) are needed to be versatile and exhibit similar levels of complexity. However, in contrast to the high number (more than 500) of PKs, there are only 107 putative Tyr phosphatases, and even less (approx. 30) Ser/Thr-specific PPs encoded in the human genome, albeit some of them have dual specificity [15]. It is therefore evident that PPs cannot be matched with PKs on a one-to-one basis; instead, since PPs are obligate multimeric enzymes, they apply a combinatorial approach in which a limited number of catalytic subunits are combined with a great variety of regulatory subunits to achieve specificity. In fact, there are only 13 genes that encode catalytic PP subunits in the human genome, which can associate with more than 100 different regulatory subunits that are the major determinants of substrate specificity and subcellular localisation [33]. Moreover, instead of sharing structural as well as operational similarities just like kinases, PPs are characterised by diverse structure, biochemistry, and even active hydrolytic sites. Based on their reaction mechanisms, Ser/Thr PPs are conventionally subdivided into three major families: (1) the aspartate-based phosphatases such as FCP/SCP which is involved in dephosphorylation of RNA polymerase II C-terminal domain; (2) metal ion-dependent (Mg^{2+} or Mn^{2+}) protein phosphatases (PPMs) such as PP2C and pyruvate dehydrogenase phosphatase; and (3) phosphoprotein phosphatases (PPs) [14, 15]. In this review article, we will now focus on the third group in the context of chondrogenesis.

PPs are responsible for dephosphorylating the vast majority of phospho-Ser/Thr residues in cells. PPs, with only a few exceptions, are multi-subunit complexes consisting of

a catalytic subunit and several regulatory subunits; each catalytic subunit is allowed to associate with a great variety of regulatory subunits to maintain specificity. The PP family of phosphatases is further subdivided into protein phosphatase 1 (PP1), PP2A, PP2B (also known as calcineurin or PP3), PP4, PP5, and PP6. For an extensive recent review, please see [16]. In the following section, individual PPs are highlighted with their distinct roles in regulating various steps of chondrogenesis, as well as signalling pathways in mature chondrocytes.

4. Protein phosphatase 1 (PP1)

Ubiquitously expressed in all eukaryotic cells, PP1 is involved in a host of cellular processes including, but not limited to, cell division, cytoskeletal rearrangement, cellular metabolism, and regulation of ion channel function [34]. Functional PP1 holoenzymes consist of a highly conserved ~35 kDa catalytic subunit (C) associated with a regulatory (R) subunit that is responsible for subcellular localisation and determines substrate specificity. While there are only a few genes (PP1cα, PP1cβ/δ, and PP1cγ) that code for catalytic subunit proteins, more than a hundred putative R subunits have been identified [35]. The naturally occurring toxins okadaic acid (OKA) and microcystin are potent inhibitors of the catalytic subunit of both PP1 and PP2A [36, 37] and are thus widely used to study the role of these phosphatases in various contexts.

4.1. Initial studies using OKA as a phosphatase inhibitor

Originally isolated from the marine sponge *Halichondria okadai*, OKA exerts inhibitory effects on PP1 and PP2A at different concentrations. During *in vitro* activity assay conditions, PP2A is completely inhibited by 1 nM OKA, whereas PP1 is unaffected at this concentration for its IC_{50} value is an order of magnitude higher (10–15 nM) [38]. Its fairly

good plasma membrane permeability makes it suitable to inhibit PP1 and/or PP2A activity in cellular systems. When 30 nM OKA was applied to cultures of primary human articular chondrocytes, a significant upregulation of cyclooxygenase-2 (COX-2) mRNA was detected, accompanied by increased COX-2 enzyme synthesis [39]. At the same time, enhanced phosphorylation of the CREB-1 transcription factor, an important co-activator of cAMP-responsive genes was observed, suggesting that PP1/PP2A may be important mediators in dephosphorylation of this protein. They also found that OKA suppressed the activity of ERK1/2, implicating that PP1/PP2A was a positive regulator of this pathway; by contrast, dramatic increase in SAPK/JNK expression and activity was identified in OKA-treated cultures of mature chondrocytes (*Fig. 1*). In that study, however, PP1 and PP2A activities have not been assayed separately, and it is therefore impossible to infer independent roles for PP1 and PP2A.

The first report on the presence and activity of PP1 in chondrogenic cells dates back to 2001 by our laboratory; in this work, OKA was applied to micromass cultures established from embryonic chick limb buds [40]. *In vitro* enzyme activity assays using ^{32}P-labelled phosphorylase revealed that PP1 activity remained steady during the course of chondrogenesis, indicating its rather general role. While administration of OKA at lower concentrations into the culture medium (2–5 nM; *i.e.* when PP1 is largely unaffected) caused slight alterations only, application at 10 or 20 nM (*i.e.* inhibition of both PP1 and PP2A could be expected based on cell-free assays according to [38]) resulted in a significant chondrogenesis-stimulating effect. However, *in vitro* PP1 enzyme activity assays revealed unchanged PP1 activity in cell lysates of cultures previously treated with 20 nM OKA. Therefore, no direct conclusions could be drawn from this study regarding the functions of PP1 during chondrogenesis. It is of note, however, that the above IC_{50} values of OKA were established in cell-free systems with purified enzymes [38], hence might not be directly

applicable to cell culture systems; *i.e.* higher concentrations might be necessary to achieve half inhibition of either PP1 or PP2A.

4.2. Further roles of PP1 in chondrocytes

In spite of its pleiotropic effects and ubiquitous expression, further data on the role of PP1 in chondrocytes is surprisingly scarce. Transforming growth factor beta (TGF-β) signalling is a well-known inducer of chondrogenesis in mesenchymal cells [41]. TGF-β elicits cellular responses ranging from proliferation, differentiation, and apoptosis through binding to their specific type I and II kinase receptors TβRI and TβRII, which in turn induce phosphorylation of specific Ser/Thr residues. Once activated, TβRI phosphorylates Smad proteins, a subclass of unique intracellular signalling molecules, which then translocate into the nucleus and regulate transcription of target genes such as *Col2a1* [42]. However, surprisingly little is known about the negative regulation of this pathway by PPs. In a study wherein a human chondrocyte cDNA library was screened in a yeast two-hybrid system, the authors demonstrated that Smad7 interacts with the growth arrest and DNA damage protein (GADD34), one of the many regulatory/targeting subunits of the PP1 holoenzyme, and that the formation of this holoenzyme mediated by TGF-β-induced Smad7 acts as a negative feedback in TGF-β signalling by dephosphorylating TβRI [43] (*Fig. 1*). These results indicate an important regulatory mechanism by which TGF-β may control the development and differentiation of various cell types, although this signalling pathway has not been verified in chondrocytes *in vivo*.

4.3. PP1 in osteoarthritis

As already mentioned, there is an urgent need for identifying novel therapeutic options for the treatment of OA. While PPs seem to be promising drug targets, their involvement in

the pathologic processes of OA has only been marginally investigated. The first study in this field established that 48-hour-long treatment with high concentrations of OKA (50 or 100 nM) elicited apoptotic cell death of OA-affected cartilage-derived human chondrocytes *via* caspase-3 activation [44]. The authors speculated that the observed effects could be attributed to PP1/PP2A inhibition; however, the extremely high concentration of OKA used in this study might have caused cytotoxic effects irrespective of PP inhibition; thus the role of PP1 in OA remains elusive.

In summary, given its pleiotropic effects and multiple targets, experimental data concerning specific roles of PP1 during chondrogenesis, as well as in mature or inflammatory chondrocytes is surprisingly scarce. This is probably because the wide array of processes with the involvement of PP1 makes it hard to study this PP using conventional techniques. *Fig. 1* summarises the known functions of PP1 in chondrogenic cells and in mature chondrocytes.

5. Protein phosphatase 2A (PP2A)

One of the most abundant cytosolic proteins, PP2A catalytic subunits can account for 0.1% of total cellular proteins in certain cell types [33]. Accordingly, PP2A is vital in regulating development, cell cycle, proliferation and cell death, mobility, cytoskeleton dynamics, and a host of intracellular signalling pathways – just like PP1. However, unlike PP1, its regulation is fairly complex. PP2A exists in two forms; a heterodimeric core enzyme and a heterotrimeric holoenzyme. The core enzyme comprises a 36 kDa catalytic (C) and a scaffold (A) subunit, each having two (α and β) isoforms. The core enzyme in turn associates with a regulatory subunit from one of the four (B, B’, B’’, B’’’) subunit families; each family can have two to five isoforms encoded by individual genes with tissue-specific expression levels. For further details on PP2A core enzymes and holoenzymes, please see [15]. As

described above, OKA is an extremely potent inhibitor of the PP2AC subunit with an inhibitory constant of 0.1 nM, which suggests that OKA is approx. 100-fold more potent for PP2A compared to PP1 [37].

Based on the above, careful selection of the effective dose of OKA may enable selective inhibition of PP2A, without influencing PP1 phosphatase activity. It is noteworthy, however, that inhibitory doses were gained from cell-free *in vitro* assays with purified enzymes (sometimes catalytic subunits only); therefore, one should be careful when drawing strict conclusions from experiments wherein OKA was applied. Following this line of reasoning, some of the observations discussed in *Section 3.1.* could be attributed to inhibition of both PP1 and PP2A (see *Fig. 1*).

5.1. PP2A regulates chondrogenesis in micromass cultures

The first papers that aimed to explore the effects of PP2A inhibition on chondrogenesis by 20 nM OKA were published by our laboratory in 2001 and 2002. Besides significantly enhancing cartilage matrix formation, which could be attributed to PP2A inhibition, application of OKA interfered with the actin cytoskeleton and a considerably higher proportion of chondrogenic cells attained a rounded, chondroblast-like phenotype [40]. In fact, PP2A has been reported to mediate actin cytoskeleton reorganisation also in fibroblasts [45]. Another interesting finding was that treatment with OKA significantly stimulated the proliferation of chondrogenic cells; although the exact mechanism remains elusive, similar effects have been observed by others (reviewed in [46]). Moreover, inhibition of PP2A by 20 nM OKA elevated the activity of PKA, and increased the phosphorylation of CREB in chicken limb bud-derived micromass cultures, suggesting that CREB was a common substrate for both the chondrogenesis-stimulating PKA and the inhibitory PP2A [47].

We have recently further dissected the involvement of PP2A in the regulation of *in*

vitro chondrogenesis and described for the first time that cyclic mechanical load reduced PP2A (and at the same time stimulated PKA) activity *via* a yet undetermined mechanism. Subsequently, the balance shifted to more strongly phosphorylated Sox9 and CREB transcription factors, and more pronounced nuclear localisation of both molecules could lie in the background of enhanced chondrogenic differentiation and matrix formation [48]. Based on these data, it is clear that PP2A can be one of the important mediators of mechanical stimuli during chondrogenic differentiation (*Fig. 2*).

5.2. Other roles of PP2A in chondrocytes

Fibroblast growth factor (FGF) is a central regulator of chondrocyte proliferation and differentiation; it inhibits chondrocyte cell division *via* the retinoblastoma (Rb) family members p107 and p130, which are essential regulators of the cell cycle [49]. The activity of Rb proteins is regulated by Ser/Thr phosphorylation; once hypophosphorylated, Rb proteins are activated and inhibit transcriptional activation *via* the E2F family of transcription factors. Conversely, phosphorylation by cyclin dependent kinases inactivates them, enabling cell cycle progression and cell proliferation to commence. Kolupaeva and co-workers described for the first time that the dephosphorylation of p107, which is a critical event in the FGF signalling pathway of chondrocytes (in rat chondrosarcoma cells) leading to cell cycle arrest, was mediated by PP2A [50]. Following up on that initial study, the same research group identified that the above effects could be attributed to the α isoform of the 55-kDa regulatory subunit (B55α) and that binding between B55α and p107 induced by FGF was a chondrocyte-specific phenomenon [51]. Furthermore, they have also shown that activation of FGF signalling leads to dephosphorylation of the B55α subunit itself on specific Ser residues (Ser125, Ser266, and Ser294) that significantly increases the affinity of the PP2A–B55α holoenzyme to p107. They speculated that either another Ser/Thr PP (for example, PP1) or

PP2A itself could mediate B55α regulatory subunit dephosphorylation. Interestingly, although with a slightly different approach, another research team has reached the same conclusion concerning dephosphorylation of p107 by the PP2A–B55α holoenzyme [52]. In their model, FGF1 signalling activates p107 dephosphorylation *via* PP2A–B55α, leading to the formation of p107 complexes to target genes regulated in the process (*Fig. 2*).

Parathyroid hormone-related peptide (PTHrP) is a well-known regulator of chondrocyte maturation. Acting through its G protein-coupled receptor, PTHrP can initiate downstream signalling that involves activation of G_s proteins, adenylate cyclase, and PKA, leading to blockage of chondrocyte maturation and hypertrophy [53]. MEF2 transcription factor is also an important element in the regulation of terminal differentiation of chondrocytes; it can induce the process by activating the transcription of *Col10a1* [54]. MEF2 is regulated by HDAC4, a member of class II histone deacetylases. In general, histone acetylation by histone acetyltransferases stimulates transcription, whereas histone deacetylation mediated by HDACs leads to transcriptional repression. HDAC4 is known to be controlled by phosphorylation at specific Ser residues, rendering it to be associated with 14-3-3 scaffold proteins, which leads to loss of repression of MEF2 activity [55]. As an attempt to shed more light on this pathway, Kozhemyakina and co-workers identified the involvement of PP2A as the phosphatase that dephosphorylates and thus enables the nuclear translocation of HDAC4. They found that in sternal chondrocytes isolated from 18-day-old chicken embryos, PTHrP signals blocked chondrocyte maturation and hypertrophy by promoting dephosphorylation of HDAC4 at Ser246 mediated by PP2A; once dephosphorylated, HDAC4 could translocate into the nucleus and repress MEF2 activity [56] (*Fig. 2*).

5.3. PP2A is differentially regulated in healthy and OA chondrocytes

As discussed above, TGF-β1 stimulates the synthesis of cartilage ECM components

including proteoglycans and collagen type II and is therefore considered as a cartilage-protective agent during inflammatory diseases such as OA. Lires-Deán and colleagues demonstrated that PP2A was differentially regulated in healthy and OA chondrocytes downstream of TGF-β1 [57]. They described that while TGF-β1 reduced the rate of apoptosis elicited by TNF-α and the PK inhibitor Ro31 in inflammatory chondrocytes, it was unable to do so in normal chondrocytes. Furthermore, while PP2A activity was not influenced by TGF-β1 in OA-affected chondrocytes, a significant increase was observed in healthy chondrocytes, implicating that TGF-β1 utilises a downstream pathway that involves PP2A in these cells, which eventually leads to a significant decrease in the bcl-2/bax ratio, indicative of apoptotic stimuli [58]. The fact that pre-treatment with the PP2A-specific inhibitor IPP2A rescued normal chondrocytes from experimentally induced apoptosis and increased the ratio of bcl-2/bax proteins suggests that PP2A could be a pivotal regulator of the anti-apoptotic effects of TGF-β1 (*Fig. 2*). These results indicate that selective modulation of the pathway that involves PP2A in inflammatory joint diseases such as OA is a promising target to be exploited. In summary, compared with PP1, more data are available pertaining to the role of PP2A in chondrogenesis and in healthy and inflammatory mature chondrocytes, but current knowledge is still far from being complete. Published data that support the function of PP2A in this field are shown in *Fig. 2*.

6. Protein phosphatase 2B (PP2B, calcineurin, PP3)

Considered as a crucial downstream effector of alterations in cytosolic Ca^{2+} concentration, calcineurin (CaN, PP2B) has been implicated in various biological processes including immune response, intracellular signalling pathways, and differentiation [59]. The CaN holoenzyme consists of a 60 kDa catalytic subunit (calcineurin A, CNA) which has at least 6 isoforms ($A\alpha_1$, splice variant $A\alpha_2$, $A\beta_1$, splice variants $A\beta_2$ and $A\beta_3$, and $A\gamma$), and a 19

kDa regulatory (calcineurin B or CNB) subunit which has 3 isoforms (B_1, a splice variant of B_1, and B_2). Due to the fact that the autoinhibitory domain is localised at the CNA subunit, CaN becomes activated only upon association with Ca^{2+}-calmodulin. The immunosuppressants FK506 (produced by the soil bacterium *Streptomyces tsukubaensis*) and cyclosporine A (CsA; isolated from the fungus *Tolypocladium inflatum*) exert their functions by inhibiting CaN after binding to immunophilins [60]. The inhibitory complexes FKBP12–FK506 and cyclophilin A (CyPA)–CsA bind to the CNB-binding helical domain of the CNA subunit. They exert their immunosuppressant effects by CaN-mediated dephosphorylation of the nuclear factor of activated T cells (NFAT). Substrates for CaN possess a consensus sequence motif of PxIxIT [61]. For further details on CaN structure and function, please see [15].

Originally identified in T lymphocytes, the NFAT family of transcription factors consists of five members (NFATc1 through NFATc4 and NFAT5), each having multiple isoforms [62]. In unstimulated cells, hyperphosphorylated NFAT proteins are localized to the cytoplasm; however, activation of calcium-dependent signalling pathways may activate CaN, which dephosphorylates NFAT, thereby exposing the nuclear localization signal that elicits translocation to the nucleus [62]. NFAT has been shown to regulate transcription of many chondrocyte-specific genes including, among others, *Col2a1* and *Acan* (reviewed in [63]). It was in that context that the first studies to reveal the presence and potential functions of CaN in chondrogenic cells and chondrocytes have been carried out.

6.1. Effects of FK506 on chondrogenesis

The first data pertaining to the effects of FK506 on chondrogenesis of the chondrogenic mouse embryonic teratocarcinoma cell line ATDC5 dates back to 2002 [60]. They found that ATDC5 cells exposed to 1 $ng \cdot mL^{-1}$ FK506 showed about a two-fold increase in Alcian blue

staining; however, CsA was unable to mimic the same effects even at higher concentrations. These results indicate that FK506 might have exerted its chondrogenesis-stimulating actions independent of CaN inhibition, albeit the authors did not aim to identify these mechanisms. Nakamura and colleagues also observed enhanced cartilage matrix accumulation, as well as increased expression of both collagen type II and type X following treatment with FK506 using the same cell line, without significant alterations in transcriptional activity of Sox6 or Sox9 [64]. The authors concluded that FK506 may promote differentiation into the proliferating chondrocyte stage through a mechanism independent of Sox9 transcriptional activity, and they were also unable to establish a direct involvement of CaN for the promotion of *in vitro* chondrogenesis in ATDC5 cells by FK506.

Following up on these initial results, Tateishi and co-workers established that FK506 (administered at 1 $\mu m \cdot mL^{-1}$) was able to promote chondrogenesis of synovial stromal cell (SSC) pellets cultured in chondrogenic medium; this effect was further enhanced with co-administration of BMP-2 and TGF-β1. Given that these two morphogens are known to mediate the Smad signalling pathway, the authors looked at whether FK506 interfered with the phosphorylation level of Smads. Indeed, levels of phospho-Smad1/5/8 and phospho-Smad3, downstream of BMP-2 and TGF-β1, respectively, were significantly higher in FK506-treated cultures than those in non-treated cells. At the same time, inhibition of Smad signalling by Noggin and SB431542 abolished FK506-induced chondrogenic differentiation of SSCs [65]. These results indicate a regulatory role of CaN in chondrogenesis *via* mediating Smad phosphorylation.

6.2. CaN is an important regulator of chondrogenic differentiation

Although the above-discussed publications failed to establish a direct link between CaN and chondrogenesis, they nevertheless suggested a negative effect of CaN during

chondrogenesis. In contrast to these observations, Tomita and co-workers were the first to show that PP2B was a positive regulator in this process. They demonstrated that in the chondrocytic mesenchymal cell line RCJ3.1C5.18, as well as in murine embryonic limb bud-derived micromass cultures, elevated cytosolic Ca^{2+} concentration elicited by ionomycin treatment enhanced chondrogenesis and matrix formation in a CaN-dependent manner; furthermore, the PP2B substrate NFAT4 was found to be hypophosphorylated in response to ionomycin. Furthermore, NFAT4 activation upregulated BMP-2 expression, which in turn induced chondrogenesis. Since the above changes could be prevented by the CaN inhibitor CsA or the BMP antagonist noggin, the authors concluded that CaN/NFAT4 signalling activates BMP-2 expression and induces *in vitro* chondrogenesis [66]; thus they provided evidence that the phosphatase involved in the BMP-2 signalling pathway could indeed be CaN, according to the assertions of Tateishi and colleagues [65].

To further characterise the involvement of CaN in growth factor-mediated signalling pathways during chondrogenic differentiation, the same research group has shown that besides BMP-2, Ca^{2+}-dependent signals also induce the expression of fibroblast growth factor-18 (FGF-18), an essential regulator of cartilage differentiation. They found that CaN and the CaN-dependent transcription factor NFAT4 were required for FGF-18 expression. FGF-18, in turn, represses noggin expression and thereby increases chondrocyte gene expression and chondrogenesis *via* BMP-dependent signalling [67]. In other words, FGF-18 and BMP-2 can cooperatively promote chondrogenesis in a CaN-dependent manner (*Fig. 3*).

The above findings established CaN as a positive regulator of chondrogenesis; however, in 2005 our laboratory was the first to demonstrate the connection between CaN activity and the master chondrogenic transcription factor Sox9. We found that CsA significantly reduced cartilage matrix formation in embryonic chicken limb bud-derived micromass cultures, and downregulated Sox9 expression. We also observed that oxidative

stress evoked by H_2O_2 had a detrimental effect on chondrogenesis that was at least partly mediated *via* inhibition of CaN and activation of ERK1/2. Moreover, CaN was found to influence the phosphorylation status of ERK1/2 and Sox9 in chicken micromass cultures; however, the exact mechanism has not been studied [26]. In a follow-up study, we observed a reciprocal regulation between alterations is cytosolic Ca^{2+} levels and CaN activity; while lower intracellular Ca^{2+} levels reduced CaN activity, application of A23187 Ca^{2+} ionophore elicited ambiguous results, suggesting the involvement of other factors in determining CaN activity. At the same time, inhibition of CaN by CsA reciprocally influenced cytosolic Ca^{2+} levels, and abolished the characteristic pattern in intracellular Ca^{2+} concentration required for chondrogenic differentiation in chicken micromass cultures [68]. Although it is unclear how CaN inhibition has led to disturbed Ca^{2+} homeostasis in chondrogenic cells, it nonetheless shows the important role of CaN during cartilage formation. More recently, we have shown that CaN can also be one of the downstream targets of pituitary adenylate cyclase activating polypeptide (PACAP) signalling in differentiating chondrocytes [48].

CaN was also demonstrated to be one of the downstream mediators of Wnt5a during chondrogenesis in mouse limb bud-derived micromass cultures. The Wnt signalling pathway plays an essential role during embryonic development; besides its pleiotropic effects, it also controls the differentiation of chondroprogenitor mesenchymal cells during chondrogenesis. While the downstream effectors of the canonical Wnts have been mapped, the signalling pathway of the non-canonical Wnt5a is less understood [69]. Bradley and Drissi has recently established that treatment of chondroprogenitor cells by Wnt5a enhanced chondrocyte maturation through activation of CaMK and CaN/NFAT-dependent induction of Sox9, with concurrent inhibition of nuclear factor-kappaB (NF-κB) [70]. The signalling pathways in which the involvement of CaN has been described to regulate chondrogenesis are summarised in *Fig. 3*.

6.4. CaN in mature chondrocytes

Since CaN unequivocally proved to be an essential positive regulator of chondrogenesis in various *in vitro* models, attempts were made to assign roles for CaN in mature chondrocytes. van der Windt and colleagues undertook to investigate CaN as a potential signalling molecule to improve chondrocyte phenotype for cartilage repair. They found that CaN activity significantly increased during dedifferentiation and decreased during redifferentiation in human articular chondrocytes *in vitro*. Moreover, inhibition of CaN activity by FK506 upregulated chondrogenic marker genes (*i.e. Col2a1*, *Acan*, *Sox9*) *via* enhanced TGF-β1 expression in dedifferentiated cells, suggesting a negative regulatory role for CaN in maintaining the mature chondrocyte phenotype in chondrocytes [71].

6.5. CaN in pathological conditions of cartilage

To exploit the potentially protective effects of CaN, several research groups have attempted to shed more light on the possible role of CaN in the pathogenesis of OA. The favourable effects of CsA on adjuvant-induced arthritis in female rats have long been known [72]; however, these effects have first been attributed to blockade of T lymphocyte activation and subsequent reduction in pro-inflammatory cytokine (mainly IL-1β) release. To establish a role for CaN in inflammatory chondrocytes, Little and colleagues undertook to analyse the effects of CsA on aggrecan catabolism mediated by matrix metalloproteinases (MMPs) in a model that mimics the early stages of OA [73]. They demonstrated that CsA inhibited aggrecanase-mediated proteoglycan catabolism elicited by administration of IL-1β in bovine articular cartilage explants; CsA was also found to downregulate several ECM catabolic factors such as ADAMTS4, ADAMTS5, MMP-13. The authors concluded that CsA may represent an effective therapeutic tool for degenerative joint diseases, without providing

implications for the involvement of CaN.

It was not until 2007 that Yoo and co-workers identified that link. They showed significantly higher levels of the catalytic subunit of CaN (CNA) in inflammatory chondrocytes, and that CsA was able to attenuate NO, MMP-1 and MMP-3 production. Furthermore, CsA treatment increased the levels of *Col2a1* and TGF-β, indicative of an augmented anabolic response. Since similar response was observed by using the natural CaN antagonist calcineurin binding protein 1, the authors speculated that CaN is a critical component in the catabolic and anabolic activities of inflammatory chondrocytes [74].

van der Windt and colleagues reached the same conclusions relating to the role of CaN in mediating anabolic and catabolic responses of chondrocytes, although from a different approach. It has long been known that during OA, the osmolality of the cartilage ECM brought about by proteoglycan depletion and collagen degradation changes from 350–480 mOsm to 280–350 mOsm [75]. In their study, they observed a significant upregulation of the chondrogenic marker genes *Col2a1*, *Acan*, and *Sox9* when OA-affected human chondrocytes were cultured at 380 mOsm with concurrent inhibition of CaN by FK506. At the same time, catabolic factors (MMPs, ADAMTS) and terminal differentiation markers (*Col10a1*, alkaline phosphatase) were repressed. Their results indicate that CaN inhibition at physiological tonicity increases the expression of anabolic markers and downregulates hypertrophic and catabolic markers [76], which perfectly aligns with the observations of other groups discussed above.

By further analysing the effects of CaN inhibition by FK506 on anabolic and catabolic markers of osteoarthritic chondrocytes in 2D and 3D *in vitro* cultures, as well as an *in vivo* rat model of OA, the same research group carried out further experiments. Besides reproducing their own results relating to induction of anabolic and reduction of catabolic ECM marker gene expressions following treatment of OA chondrocytes by FK506, they also reported that

inhibition of CaN activity by FK506 treatment *in vivo* reduced OA-like responses (*i.e.* cartilage ECM degradation, macrophage activation, osteophyte formation) in articular joint tissues. As a main conclusion, the authors implicate CaN as an interesting target for future therapeutic management of OA [77].

In summary, PP2B is known to be involved in multiple aspects of chondrogenesis, and it also plays an important role in the balance between anabolic and catabolic changes in mature chondrocytes. *Fig. 3* depicts the known functions of CaN during chondrogenesis, whereas *Fig. 4* summarises its roles in mature and/or OA-affected chondrocytes.

7. PP4, PP5, and PP6 have not been studied in chondrogenesis

Apart from PP1, PP2A, and PP2B, the potential roles of other phosphoprotein phosphatases (PP4, PP5 and PP6) have not been investigated either in differentiating or mature chondrocytes. *PP4* (also known as PPX) is closely related to PP2A in that it also consists of catalytic and regulatory subunits, and it also forms heterodimeric core enzymes and heterotrimeric holoenzymes. An essential phosphatase in all eukaryotic species, PP4 has been reported to be involved in centrosome duplication, apoptosis, and DNA repair; regulates the JNK pathway; and mediates the effects of TNF-α [78]. In spite of its fundamental roles and the fact that is has been described more than 20 years ago [79], PP4 has not previously been shown either in differentiating or in mature chondrocytes. PP4 is known to govern differentiation of neural stem and progenitor cells, furthermore *Smek*, a regulatory subunit of PP4 has been recently demonstrated to play an important role in histone deacetylation and silencing of the Wnt-responsive gene *brachyury* in embryonic stem cells [80]. As already discussed, the Wnt pathway is an essential regulator of chondrogenesis; in particular, Wnt5a enhances chondrocyte maturation in a CaN-dependent manner [70]. Lyu and colleagues suggested that a Smek-containing PP4 complex represses the transcription of Wnt-responsive

genes through histone deacetylation, and that this complex is essential for the maintenance of embryonic stem cell pluripotency [80]. Following this line of reasoning, it would be interesting to analyse whether PP4 regulates chondrogenesis *via* modulation of Wnt signalling.

Originally described in 1994, *PP5* is a rather exotic phosphatase in that it is encoded by a single gene in all eukaryotic organisms, and that the regulatory and catalytic domains are contained within a solitary polypeptide [81]. Expressed in all tissues examined, PP5 regulates a diverse array of functions including proliferation, differentiation, migration, and stress-induced signalling [15]. Still, no studies have been conducted to assign specific roles to this PP in differentiating or mature chondrocytes. According to the paper of Chen and co-workers, PP5 negatively regulates embryonic stem cell differentiation by maintaining *Nanog* expression through the inhibition of ERK signalling [82]. *Nanog* has been reported to be expressed also in chondrogenic micromass cultures [83]; therefore, it would be interesting to find a connection between *Nanog* expression, PP5, and chondrogenic differentiation. Furthermore, since PP5 is known to mediate stress-induced signalling, it might have important implications in degenerative cartilage disorders such as OA. This theory, however, is yet to be confirmed.

The catalytic subunit of *PP6*, another essential phosphatase, is also closely related to that of PP2A. PP6 is described to form a heterotrimeric holoenzyme that consists of a catalytic subunit, a Sit4-related scaffold subunit, and an ankyrin repeat-containing subunit as a regulatory subunit. Although first described in 1989 [84], relatively little is known about this enzyme except for its role in progression through the cell cycle [16]. Further studies are needed to characterise specific roles of PP6 in chondrocytes.

8. Conclusions and perspectives

Although the number of genes that encode the catalytic subunits of PPs is much smaller compared to the number of genes that encode Ser/Thr PKs, current knowledge relating to the function of PPs is scarce, especially in the field of chondrogenesis. The combinatorial nature of PPs and the resultant complexity could have hindered research progress in this field; indeed, the wide range of different regulatory subunits and other interacting proteins that can associate with PP1 or PP2A catalytic subunits confer different substrate specificity, subcellular localisation, and regulation, eliciting sometimes opposing effects on signalling pathways. Besides the enormous complexity of PP1 and PP2A holoenzymes, there are some other unique features of PPs that hamper their studies. For instance, levels of PP catalytic subunits tend to be relatively constant in cells and resist alteration by either overexpression or knockdown by small interfering RNA techniques to preserve function. Furthermore, since PPs possess a robust catalytic activity, even a small quantity of the functional protein is sufficient to exert substantial effects [16].

Based on the above specific features, current understanding of PP function in differentiating and mature chondrocytes is far from complete. There are major unanswered questions regarding the specific roles for every PP family or subfamily; for example, how exactly do they balance the effects of PKs during chondrogenesis, and what functions do they play in pathological processes of cartilage. One of the major challenges is to identify the substrate proteins for PPs; in other words, to determine which phosphatase is responsible for dephosphorylation of a given phospho-protein in differentiating chondrocytes. Because of the obstacles outlined above, the classic approach that employs the application of more or less selective PP inhibitors and/or overexpression/gene silencing will likely fail. Phosphoproteomics may provide a versatile tool in assigning phosphatases to substrates in chondrocytes. This approach has been successfully applied to detect phosphorylation pattern following mechanical stretching in chondrosarcoma cells [85]. By better exploiting the

sensitivity of this approach, a more detailed discovery of PP substrates is envisaged.

8.1. Regulation of ion channel functions and Ca^{2+} homeostasis by PPs

Among the most interesting candidate targets for PPs are ion channels. Although considered as an archetypal non-excitatory cell, the chondrocyte employs a full complement of a great variety of ion channels to maintain cellular ion homeostasis (for a review, see [86]). Moreover, not only are they essential components of maintaining ion homeostasis of mature chondrocytes but they are also required for chondrogenic differentiation. Various ion channels including TRPV4 [87]; the ionotropic purinergic receptor $P2X_4$ [7, 88]; chloride channels [89]; voltage-gated sodium and potassium channels [8]; and voltage-dependent Ca^{2+} channels [5] have been described to be involved in chondrogenesis. Besides the important role of cytosolic Ca^{2+} in differentiating chondroprogenitors and subsequent activation of Ca^{2+} dependent signalling cascades such as PKC or PP2B, it would be intriguing to investigate if phosphorylation in turn mediates cytosolic Ca^{2+} levels *via* modulation of ion channel activity in chondrocytes.

In 1989, Armstrong postulated that there was a close interaction between CaN, PKA, and dihydropyridine-sensitive voltage-gated Ca^{2+} channels (*i.e.* $Ca_V1.1$) in which CaN, by dephosphorylating the Ca^{2+} channel, would inactivate it and the reduced Ca^{2+} influx would in turn deactivate CaN [90]. This hypothesis was confirmed with *de facto* electrophysiological data by Fomina and Levitan who demonstrated that in rat pituitary lactotrop cells inhibition of CaN activity by CsA has led to reduced ionic currents *via* voltage-gated Ca^{2+} channels [91]. Since then, CaN has been shown to modulate a number of ion channels including inhibition of the arachidonate-regulated Ca^{2+} channels (ARC channels that are closely related to the store-operated CRAC channel Orai proteins) [92]; the intracellular Ca^{2+} release channels inositol trisphosphate receptor (IP_3R) and ryanodine receptor (RyR) [61]; or the

vanilloid receptor TRPV1 [93]. In smooth muscle cells, PP1 was described to increase the open probability of the large-conductance, voltage- and Ca^{2+}-gated K^+ (BK_{Ca}) channel; in contrast, PP1 inhibited the A-type K^+ channel 19-pS in mouse colon cells through its fast inactivation mechanism. Apart from potassium channels, PP1 has been reported to be involved in activating and/or deactivating L-type Ca^{2+} channels (for a review, see [94]). PP2A has also been implicated to regulate L-type Ca^{2+} channel and BK_{Ca} K^+ channel function [94].

However, none of these interactions have been demonstrated either in differentiating or in mature chondrocytes. In particular, our own laboratory supplied indirect data to support the idea that CaN can in fact influence Ca^{2+} ion channel activities during chondrogenesis; application of CsA to differentiating chondroprogenitors eliminated the peak in basal cytosolic Ca^{2+} concentration, suggesting an important regulatory role for CaN [68]. Future studies are required to elucidate the precise involvement of CaN and other PPs in dephosphorylating specific ion channels during the course of chondrogenesis.

We have recently documented that in parallel to Ca^{2+} influx, Ca^{2+} release from internal Ca^{2+} stores and subsequent store refilling through store-operated Ca^{2+} entry (SOCE) mediated by Orai and STIM molecules was also required for chondrogenesis to take place [5]. Although considerable research has been done in that field during the last couple of years, the posttranslational regulation of the molecules that orchestrate SOCE is still elusive. It has only recently been shown that CaN, associated with kinase suppressor of ras 2 (KSR2), was indispensable for SOCE; CaN inhibition impaired formation of STIM1/Orai1 puncta and cytoskeleton organization [95].

While long-term sustained Ca^{2+} signals are undoubtedly essential for chondrogenic differentiation, high-frequency Ca^{2+} oscillations mediated by voltage-dependent K^+ and Ca^{2+} channels, as well as SOCE, are also of particular importance [5, 8]. Pulsatile changes in local

cytosolic Ca^{2+} concentration have long been known to modulate a host of cellular processes including gene transcription. NFAT is one of the oscillation-sensitive transcription factors; in fact, besides CREB [96], NFAT has been proposed to be one of the key mediators and molecular decoders of information encoded in the frequency of Ca^{2+} spikes [97]. Given that CaN is responsible for the Ca^{2+}-dependent dephosphorylation and nuclear translocation of NFAT, it seems plausible that Ca^{2+}-dependent activation of NFAT is attributable to CaN. In contrast, stimulation with agonist concentrations that elicited oscillatory Ca^{2+} signals failed to increase CaN activity [92]. Nevertheless, CaN activation is clearly context-dependent and differentiating chondroprogenitors might represent a cell type in which CaN could be one of the key molecular regulators of cytosolic Ca^{2+} fluctuations. Taken together, it would be interesting to link CaN and other phosphatases to the tight control of SOCE, Ca^{2+} influx through plasma membrane ion channels, and the dynamic changes of global Ca^{2+} homeostasis during chondrogenesis.

Such a link has been established in a recently published paper by Lin and colleagues [98]. They documented that Ca^{2+} influx through the T-type voltage-operated Ca^{2+} channel $Ca_V3.2$ was essential for chondrogenesis in tracheal cartilage (but not in articular cartilage), without modulating the proliferation of differentiating mesenchymal cells. They described that Ca^{2+} influx through $Ca_V3.2$ activated the CaN/NFAT signalling and upregulated cartilage ECM-specific marker genes such as *Sox9, Acan* and *Col2a1*, since treatment with CsA eliminated the observed effects. Moreover, they identified an NFAT-binding motif (TTTCC) in the mouse Sox9 promoter, which provides direct evidence for the first time that the Ca^{2+}–CaN–NFAT axis can indeed upregulate Sox9 expression during chondrogenesis [98]. These results are in a perfect agreement with our own data that T-type voltage-dependent Ca^{2+} channels, including $Ca_V3.2$, were expressed in chondrifying micromass cultures, and that blockade of these channels detrimentally affected chondrogenesis [5].

8.2. Osteoarthritis and protein phosphatases – candidate drug targets?

As discussed above, PP1, PP2 and PP2B have all been studied in the context of OA. It must be noted, however, that available data are surprisingly scarce, and thus we are only at the beginning to understand the role of PPs during the pathogenesis of OA. Nevertheless, despite the small number or relevant studies, it seems to be the case that PPs, CaN in particular, appears to be an interesting target for future therapeutic management of OA. Given the pleiotropic effects and essential functions of PPs in virtually every cell in the body, it will be challenging to find solutions that offer targeted delivery and reduce undesirable side effects. For example, chronic systemic treatment with the CaN inhibitor FK506 is known to have adverse effects on many tissues and organs including nephrotoxicity and neurotoxicity [99]. At the same time, local intra-articular delivery of FK506 may increase the risk of iatrogenic arthritis [77]. Thus, there is a pressing need to develop novel, more specific drugs that target PPs in a context-dependent manner in OA-affected articular cartilage, ideally without adverse effects that would advance disease progression. Obviously, these proteins are very difficult to target for drug development. The challenge should be easier for PP1 and PP2A by developing regulatory subunit-specific inhibitors that would exert their disease-modifying effects in OA-affected cartilage only. Local (intra-articular) administration of such novel drugs may facilitate efficacy and at the same time reduce the risk of systemic adverse effects.

In conclusion, the published scientific data reviewed in this paper clearly indicate that PPs are indeed important regulators of differentiating and mature chondrocytes, with important implications for disease-specific functions. Yet there are many unanswered questions. For protein phosphorylation to be reversible, a delicate balance between PKs and PPs must exist at all times. While multiple PKs involved in the highly complex signal

transduction and other physiological systems that control the molecular machinery of chondrogenesis are confirmed, only a very small portion of PPs have been mapped. As opposed to PKs where at least a few important functions of all major families have been established, PPs have only been described in a much more restricted context. Clearly there is an urgent need for studies with a primary aim to elucidate novel aspects and assign novel functions to the neglected yet essential components of reversible protein phosphorylation during chondrogenesis.

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Conflict of Interest Statement

The authors wrote this review within the scope of their academic and affiliated research positions. There was no bias or external involvement in this work and the authors declare no competing interests. The authors do not have any commercial relationships that could be construed as biased or inappropriate.

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Table 1. Roles of the main PK families in controlling the key steps in chondrogenesis and signalling pathways in chondrocytes.

PK family	**Role(s)**	**Reference***
cyclic AMP dependent protein kinase (PKA)	Positive regulator of chondrogenesis; mediates BMP-2 signalling and phosphorylates CREB; modulates N-cadherin expression *via* activating PKCα	[17, 18, 20]
Protein kinase C (PKC) isoenzymes	Positive regulators of chondrogenesis; mediate the expression of cell adhesion molecules, actin cytoskeleton reorganisation, modulates MAPK pathway	[6, 19]
Mitogen-activated protein kinases (MAPKs)		[3]
1. Extracellular signal-regulated kinase (ERK1/2)	Negative or positive regulator of chondrogenesis, depending on embryological origin, differentiation stage, and concurrently active other pathways; mediates the effects of BMP-2, FGF, IGF-1, etc.	[20, 23, 25]
2. p38	Positive regulator of chondrogenesis; mediates the effects of BMP-2, TGF-β2, etc.	[22]
3. c-jun N-terminal kinase (JNK)	Positive or negative regulator of chondrogenesis; mediates the effects of Gas6; modulates miR-34a	[27, 28]
Ca^{2+}/calmodulin-dependent protein kinases (CaMKs)	CaMKII is involved in mechanotransduction; modulate hypertrophic gene expression	[31, 32]

*For simplicity, only a few selected references are shown. Where available, the reader is referred to recent relevant review articles for further details

Figures

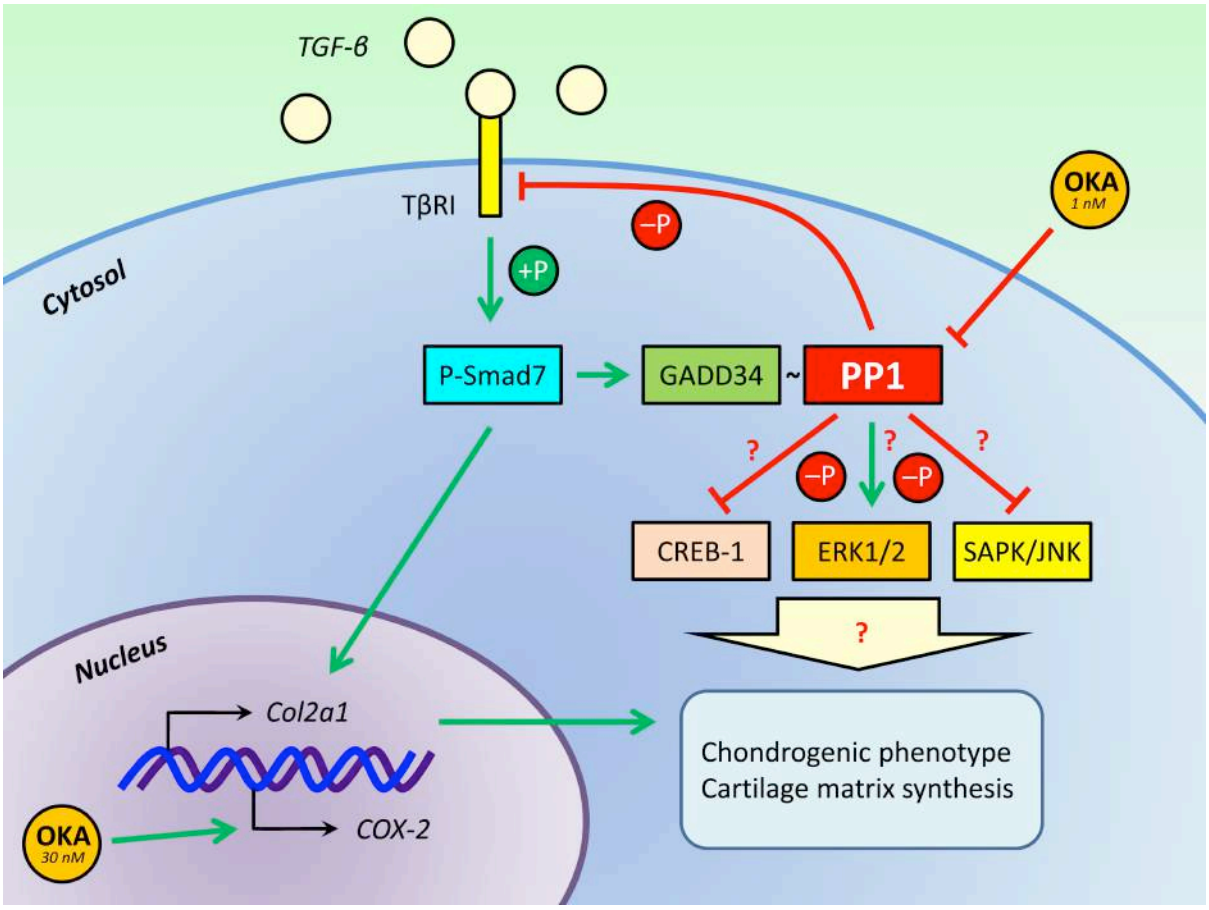

Fig. 1. PP1 appears to play a negative regulatory role in differentiating chondrocytes. Available data suggest an inhibitory function for PP1; it dephosphorylates CREB; activates ERK1/2 signalling; attenuates the JNK pathway; and dephosphorylates TβRI, and thus blocks the TGF-β–Smad7–*Col2a1* pathway in chondrocytes. For further details and references, please see text. Noteworthy, however, that depending on the concentration, OKA is a potent inhibitor also of PP2A, therefore one should be cautious when drawing conclusions from these potentially ambiguous results. (CREB-1, cAMP response element binding protein-1; COX-2, cyclooxygenase-2; ERK1/2, extracellular signal-related kinase 1/2; GADD34, growth arrest and DNA damage protein-34; OKA, okadaic acid; SAPK/JNK, stress-activated protein kinase/jun N-terminal kinase; TGF-β, transforming growth factor-beta; TβRI, TGF-β receptor I)

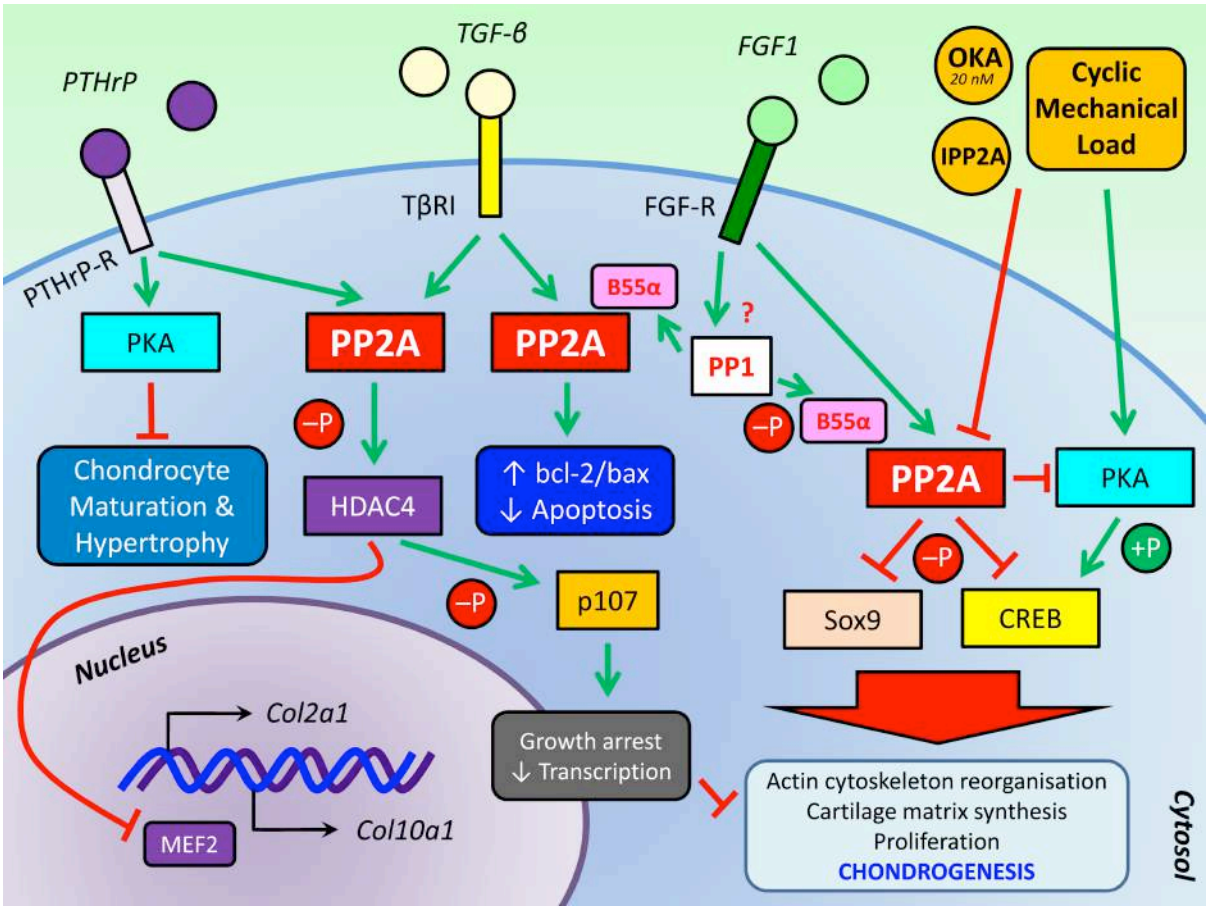

Fig. 2. PP2A exerts a negative role in chondrogenesis and in differentiating chondrocytes. Inhibition of PP2A activity by 20 nM OKA (or IPP2A, or cyclic mechanical load) promotes chondrogenesis and cartilage ECM production, and enhances proliferation. PP2A reduces Sox9, CREB and PP2A activity during chondrogenesis. PP2A is involved in the downstream signalling of FGF1, TGF-β, and PTHrP. The 55-kDa regulatory subunit (B55α) is known to be involved in certain pathways. PP2A seems to negatively regulate maturation and hypertrophy by dephosphorylating HDAC4. PP2A is also reported to regulate the cell cycle *via* p107 and modulate apoptosis. For further details and references, please see text. (CREB, cAMP response element binding protein; ERK1/2, extracellular signal-related kinase 1/2; FGF1, fibroblast growth factor-1; HDAC4, histone deacetylase-4; MEF2, myocyte enhancer factor-2; OKA, okadaic acid; PKA, protein kinase-A; PTHrP, parathyroid hormone related peptide; TGF-β, transforming growth factor-beta; TβRI, TGF-β receptor I)

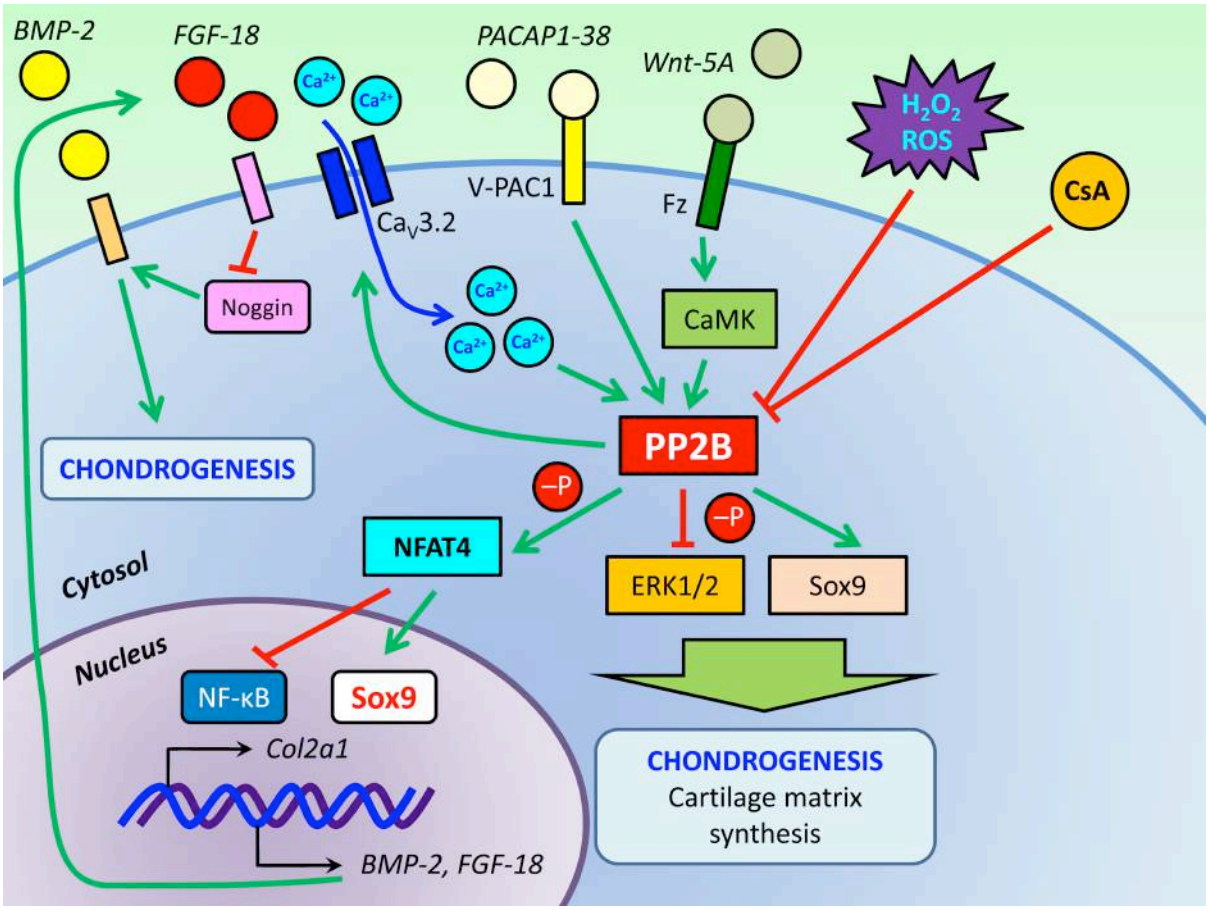

Fig. 3. CaN is an important chondrogenesis-stimulating regulator during chondrogenesis. Responses to multiple soluble factors including Wnt-5A, PACAP1–38, FGF-18, and BMP-2 are known to be mediated *via* CaN. Local or global increases in cytosolic [Ca^{2+}] are also required for CaN activation. CsA and ROS (H_2O_2) can inhibit CaN activity. One of the main downstream targets is NFAT-4 which, after CaN-mediated dephosphorylation, translocates into the nucleus and upregulates cartilage-specific genes. CaN also activates Sox9, but inhibits the ERK1/2 pathway. CaN is implicated to regulate ion channels and thus modulate ion conductivities. References and further details are in the main text. (BMP-2, bone morphogenic protein-2; CsA, cyclosporine A; ERK1/2, extracellular signal-related kinase 1/2; FGF-18; fibroblast growth factor-18; NFAT-4, nuclear factor of activated T lymphocytes-4; NF-κB, nuclear factor-kappa B; PACAP, pituitary adenylate cyclase activating polypeptide; ROS, reactive oxygen species)

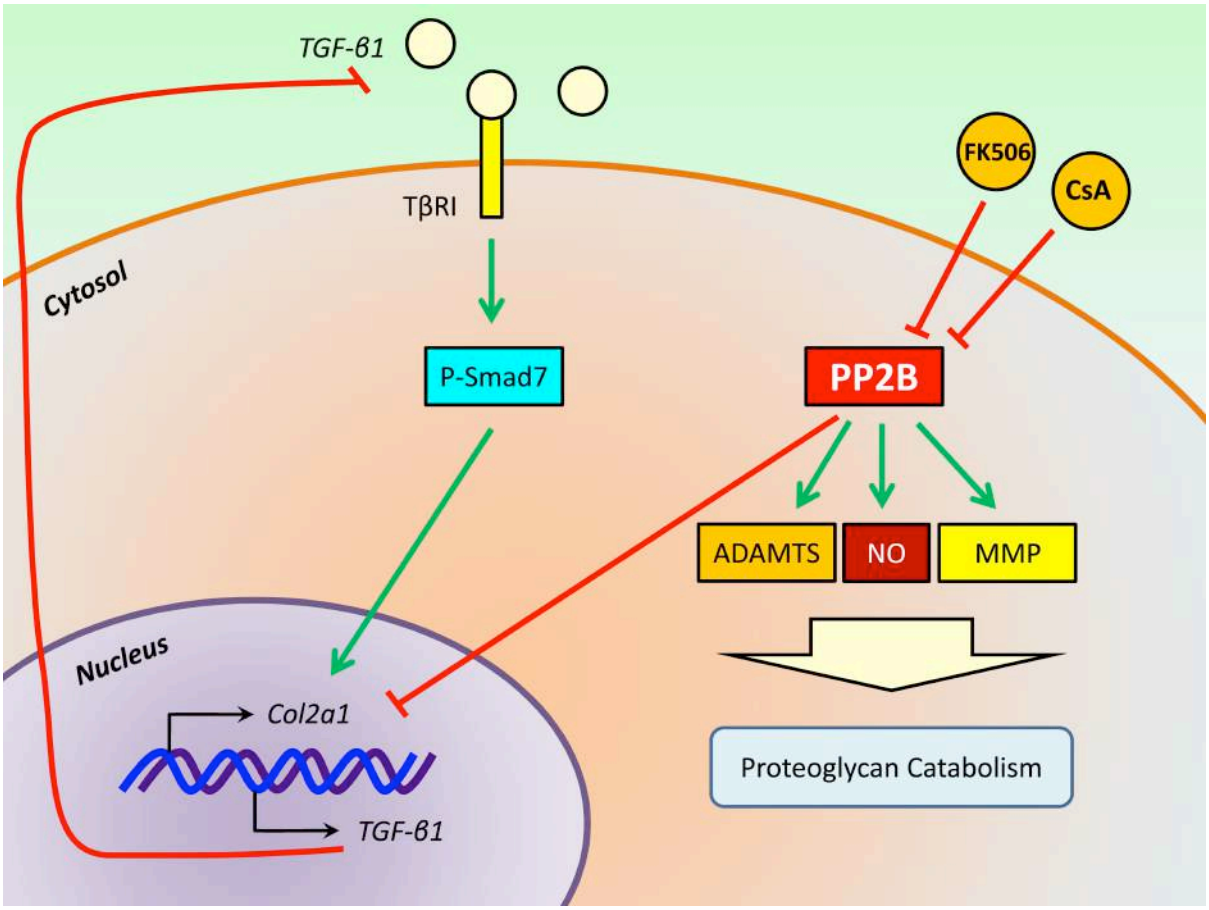

Fig. 4. CaN negatively regulates the chondrocyte phenotype in mature or OA-affected chondrocytes. In contrast to chondrogenesis, CaN activates catabolic enzymes including ADAMTS, MMP, as well as NO, and blocks transcription of cartilage-specific genes (such as Col2a1) and TGF-β1. See text for more detailed explanation and relevant references. (ADAMTS, "A Disintegrin And Metalloproteinase with Thrombospondin Motifs"; CsA, cyclosporine A; MMP, matrix metalloprotease; NO, nitrogen oxide; TGF-β1, transforming growth factor beta-1; TβRI, TGF-β receptor I)