Review

Regulation of chondrogenesis by protein kinase C: Emerging new roles in calcium signalling

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ABSTRACT

During chondrogenesis, complex intracellular signalling pathways regulate an intricate series of events including condensation of chondroprogenitor cells and nodule formation followed by chondrogenic differentiation. Reversible phosphorylation of key target proteins is of particular importance during this process. Among protein kinases known to be involved in these pathways, protein kinase C (PKC) subtypes play pivotal roles. However, the precise function of PKC isoenzymes during chondrogenesis and in mature articular chondrocytes is still largely unclear. In this review, we provide a historical overview of how the concept of PKC-mediated chondrogenesis has evolved, starting from the first discoveries of PKC isoform expression and activity. Signalling components upstream and downstream of PKC, leading to the stimulation of chondrogenic differentiation, are also discussed. Although it is evident that we are only at the beginning to understand what roles are assigned to PKC subtypes during chondrogenesis and how they are regulated, there are many yet unexplored aspects in this area. There is evidence that calcium signalling is a central regulator in differentiating chondroprogenitors; still, clear links between intracellular calcium signalling and prototypical calcium-dependent PKC subtypes such as PKCalpha have not been established. Exploiting putative connections and shedding more light on how exactly PKC signalling pathways influence cartilage formation should open new perspectives for a better understanding of healthy as well as pathological differentiation processes of chondrocytes, and may also lead to the development of novel therapeutic approaches.

Contents

1. Introduction

Cells of multicellular organisms are constantly exposed to the extracellular environment and receive a plethora of chemical signals at the same time. Among these signals are various endocrine, paracrine, juxtacrine and autocrine factors including hormones (*e.g.* somatotropin and insulin); cytokines, growth and differentiation factors such as bone morphogenic proteins (BMPs), epidermal, fibroblast or insulin-like growth factors (EGF, FGF, IGF), or transforming growth factors (TGFs); as well as morphogens, *e.g.* retinoic acid, Wingless (Wnt), sonic hedgehog (Shh), or indian hedgehog (Ihh). These molecules are bound to plasma membrane or intracellular receptors and interpreted by complex molecular pathways that utilise specific combinations of a cell or tissue-specific signalling toolkit, and by eventually converging on transcription factors they often induce changes in gene expression. These signals are required to adjust the cellular metabolism to the needs of the tissue and/or organism, or to determine decisions that affect the fate of cells: proliferation, differentiation, or apoptosis [1].

This is particularly true for differentiating embryonic tissues, where individual cells are required to act in an extraordinarily co-ordinated way both temporally and spatially to ensure all tissue and cell types are formed appropriately. This process is known as specification and differentiation, which involves the expression of unique cellular functions that enable individual cells to contribute to the operation and needs of different tissues, organs and organ systems [2]. For differentiation to take place, a carefully orchestrated sequence of events is required that is tightly regulated by complex signalling pathways.

2. Chondrogenesis is regulated by a complex interplay between many factors

Cartilage formation, one of the earliest morphogenetic events during embryonic development, is a highly organised multistep process that involves condensation of chondroprogenitor cells, differentiation into chondroblasts and then chondrocytes, and the transformation of chondrogenic tissues into skeletal structures (recently reviewed by [3]). The process is characterised by substantial changes in the shape of chondroprogenitor cells, which requires transition to a spherical chondroblast and chondrocyte-like morphology from the elongated fibroblast-like shape [4]. Although the main steps of chondrogenesis that takes place in embryonic limb bud-derived micromass cultures

have been partially mapped, there are still many yet unresolved questions. *De facto* chondrogenesis is preceded by the appearance of precartilaginous condensations, brought about by enhanced proliferation and active migration of chondroprogenitors; these condensations enable the establishment of cell–cell and cell–matrix interactions (*via* gap junction, N-cadherin and N-CAM and through integrins, respectively) that initiate inter- and intracellular signalling events [5]. Apart from cell adhesion molecules, interactions of various proteins including FGFs, TGFs, BMPs, Wnt, Shh, and products of the homeobox (Hox) genes are also required [6]. Acting through their receptors, these factors activate their downstream targets that are essential for initiation and maintenance of the chondrocyte phenotype.

Apart from soluble factors, macromolecules of the developing extracellular matrix (ECM) itself including collagen type II, hyaluronan, the large aggregating proteoglycan (PG) aggrecan, or fibronectin can also act as signalling molecules [6]. For example, binding of integrin α5β1 (the fibronectin-receptor) to its ligand initiates the establishment of focal adhesions and leads to the activation of focal adhesion kinase (FAK), which in turn interacts with the phosphoinositol-3 kinase (PI-3K) and contributes to the activation of mitogen-activated protein kinases (MAPK) [7]. The MAPK cascades play very important roles in mediating the effects of extracellular signals to regulate a variety of cellular functions, including proliferation, differentiation, and stress responses. It has long been known that various MAPKs including the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 regulate chondrogenesis in chicken limb bud-derived micromass cultures; while ERK1/2 was found to be a negative regulator, p38 is a positive regulator of chondrogenesis as it modulates the expression of cell adhesion molecules (*e.g.* N-cadherin, and integrin α5β1) at the post-precartilage condensation stages [8]. Besides ERK1/2 and p38, the involvement of the third MAPK pathway, the c-Jun N-terminal kinase (JNK) signalling pathway, was also reported recently in the differentiation of chondrocytes [9,10].

The cartilage-specific transcription factors Sox9, L-Sox5 and Sox6 that are often referred to as the Sox-trio are essential for the expression of ECM proteins (*e.g. COL2A1*, aggrecan core protein). The Sox transcription factors, Sox9 in particular, are strongly expressed throughout chondrogenesis as they are required for the initial steps of condensation and for maintaining the chondroprogenitor phenotype of differentiating mesenchymal cells [11]. Since the Sox9 transcription factor is indispensable to chondrogenesis, many signalling pathways are known to converge on

regulating Sox9 expression and/or function. Furthermore, although the above-described pathways are central components of chondrogenesis, their regulation is accomplished by other factors, such as Ser/Thr protein kinases.

Protein kinase A (PKA) is among the earliest known regulators of chondrogenesis; Solursh and co-workers reported that cyclic adenosine monophosphate (cAMP) levels increased during the initial condensation of mouse and chick limb bud-derived mesenchymal cells cultured *in vitro* [12]; furthermore, exogenous cAMP derivatives were shown to enhance *in vitro* chondrogenesis [13]. These discoveries have led to establishing a chondrogenesis-promoting role for PKA, which does not only mediate the positive effects of BMP on chondrogenic differentiation by phosphorylating Ser/Thr residues of key substrates [14] but also regulates cAMP responsive genes *via* the cAMP response element binding protein (CREB) that binds to CRE sites within promoters of certain genes; for example, the Sox9 promoter itself is known to be regulated by CREB [15]. Besides controlling its expression, PKA is also known to phosphorylate the Sox9 protein (at Ser 211), which induces its translocation into the nucleus and enhances its transcriptional activity [16].

Apart from various protein kinases, phosphoprotein phosphatases (PP) that counterbalance the effects of kinases are equally important regulators of chondrogenesis. Our laboratory was the first to demonstrate that PP2A negatively regulates chondrogenic differentiation as its inhibition with okadaic acid enhanced chondrogenesis *via* modulating proliferation and cytoskeletal organisation in mesenchymal cells, as well as through cross-talk with the PKA signalling pathway [17]. Furthermore, CREB was identified as one of the important common targets for the PP2A and PKA pathways whose phosphorylation status was modified according to the actual balance between the two signalling mechanisms [18]. We have recently demonstrated that cyclic mechanical load exerts chondrogenesis-stimulating effects *via* opposing regulation of PKA and PP2A signalling in micromass cultures of embryonic chicken limb bud-derived differentiating chondroprogenitors [19]. The Ca^{2+}–calmodulin dependent PP2B (calcineurin), on the other hand, is a positive regulator of chondrogenesis in chick limb bud-derived micromass cultures, which exerts its chondro-stimulatory effects *via* the ERK1/2 pathway [20].

Signalling events including extracellular soluble factors and cell junction molecules that are reported to control various steps of chondrogenesis including condensation and changes in cell morphology are shown in Fig. 1.

3. Protein kinase C: a versatile family of Ser/Thr kinases

Indeed, there is a complex synergism between signalling pathways that control the molecular steps of chondrogenesis. Yoon and co-workers reported that in chondrifying micromass cultures of embryonic limb bud-derived mesenchymal cells, PKA regulates chondrogenesis by activating PKCα [21]. Unlike PKA, PKC is a multigene family that comprises a group of highly related Ser/Thr protein kinases, which were first identified almost 40 years ago by Nishizuka and colleagues from bovine cerebellum [22]. PKC isoenzymes are highly conserved in eukaryotes, ranging from a single isoform in the yeast (*S. cerevisiae*) to 12 isoforms in mammals and thus constitute ~2% of the human kinome [23]. All PKC isoforms possess a highly conserved kinase domain at the C-terminus that is linked to a much more divergent regulatory domain at the N-terminus by a flexible hinge region. Furthermore, all PKC isoenzymes contain a pseudosubstrate sequence that is present in their regulatory domain; when the enzyme is inactive (auto-inhibited), the pseudosubstrate region occupies the substrate-binding pocket in the kinase domain. When PKC is activated, the appropriate second messengers bind to the regulatory domain, displacing the pseudosubstrate region from the active site. For relevant recent reviews, see [24] and [25].

Based on their regulatory domains and activation mechanisms, the PKC family can be divided into four structurally and functionally distinct subgroups (Fig. 2). The *classic PKCs (cPKCs)* comprise PKCα, PKCβ and PKCγ; these enzymes are activated by binding of diacylglycerol (DAG) and phospholipids to their C1 domains, as well as Ca^{2+}-dependent binding of phospholipids to their C2 domains. The *novel PKCs (nPKCs)*, which include PKCδ, PKCε, PKCη and PKCθ, are similarly activated by DAG and phospholipids, but they do not require Ca^{2+}. The *atypical PKCs (aPKCs)* comprise PKCι/PKCλ (the latter in mice) and PKCζ; they do not require Ca^{2+} or DAG for their activation. Just like aPKCs, members of the *PKN subfamily* (PKN1, PKN2 and PKN3) are allosterically activated by their partners *via* interactions with specific motifs and domains; for a review, see [26]. The fact that conventional PKCs require Ca^{2+} for their activation has significant consequences, as these enzymes may be pivotal mediators of global as well as localised changes in cytosolic Ca^{2+} levels [27]. In addition to these methods of activation, other regulatory processes can influence the function of PKCs, including phosphorylation. The varied mechanisms of activation, as well as the modular nature of the PKC family allow PKC activity to be deployed with versatile spatial and temporal specificity. As a result, the PKC
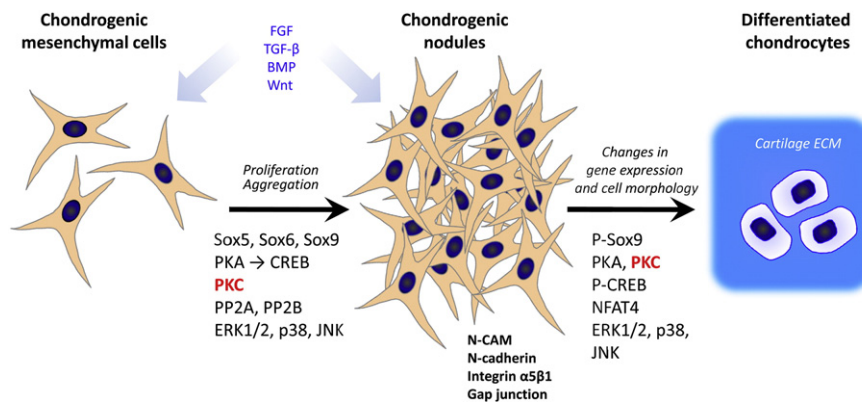
Fig. 1. Main steps of chondrogenesis and the signalling pathways involved in its regulation. Chondroprogenitor cells are initially loosely arranged with elongated mesenchymal cell morphology. During condensation, chondrogenic cells undergo extensive proliferation and migration, resulting in the formation of precartilaginous nodules. Soluble factors (such as FGF, TGF-β, BMP, Wnt), cell adhesion molecules (N-CAM, N-cadherin, integrin α5β1) and intracellular signalling molecules including ERK1/2, p38 and JNK MAPKs, PKA, PP2A and PP2B, as well as PKC are required for this step. After the differentiation process has occurred, cells gain round morphology as a result of cytoskeletal rearrangement and acquire a gene expression pattern required for cartilage-specific ECM production and maintenance. In each step of chondrogenesis, PKC isoforms play essential roles in the signalling pathways. For details, please see text. (FGF: fibroblast growth factor; TGF-β: transforming growth factor beta; BMP: bone morphogenic protein; PKA, PKC: protein kinase A and C; PP2A and PP2B: phosphoprotein phosphatase 2A and 2B; ERK1/2: extracellular signal regulated kinase; JNK: c-jun N-terminal kinase; CREB: cAMP-responsive element binding protein; NFAT: nuclear factor of activated T-lymphocytes).
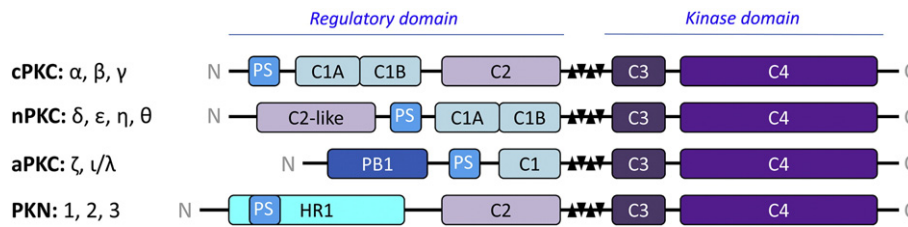
Fig. 2. Schematic illustration of the domain structure of the PKC superfamily. PKCs consist of a conserved kinase domain (towards the C-termini) and more variable regulatory domains (towards the N-termini) with a flexible hinge region between them. PKC isoenzymes can be divided into four structurally and functionally distinct subgroups according to their regulatory domains: classical isoforms (cPKC), novel isoforms (nPKC), atypical isoforms (aPKC) and the PKC-related kinases (PKN). Conserved region 1 (C1; shown in grey) can confer binding of DAG and phospholipids; C2 (in light purple) functions as a Ca^{2+}-dependent phospholipid binding module. Note that the C2 domain in nPKC does not bind Ca^{2+}. Tandem C1 domains (C1A and C1B) are molecular sensors of PMA/DAG in cPKC and nPKC isoforms, whereas the single aPKC C1 domain does not bind DAG/PMA. Phox/Bem domain 1 (PB1; in dark blue) acts as a dimerisation site with various proteins. Homology region 1 (HR1; in light blue) that is only present in PKN confers small-GTPase binding properties. PKCs are regulated by auto-inhibition through their pseudosubstrate sites (PS; in ultramarine) that binds to the substrate-binding pocket in the C4 kinase domain (in purple). When the regulatory domain is recruited to the plasma membrane through Ca^{2+}, phospholipid and DAG, autoinhibition is relieved.
Adapted from [25] and [26].

family is centrally involved in the spatial and temporal controls of signal transduction pathways in a plethora cell types. PKC contributes to the regulation of almost every aspect of cells, ranging from migration, secretion, cell–cell connections, polarity, cell proliferation and cell death, gene transcription and translation, cell morphology, regulation of ion channels and receptors, and differentiation [24].

4. PKC is a quintessential regulator of chondrogenesis

### 4.1. Early results with administration of phorbol esters or PKC inhibitors

Initially, the majority of studies aiming at identifying the role of PKC enzymes in chondrogenesis involved the use of tumour promoter phorbol esters (*e.g.* phorbol-12-myristate-13-acetate, PMA). By mimicking DAG and binding to the C1 domain, these compounds are potent activators of PKC. However, as PMA irreversibly activates PKC isoenzymes, it does not perfectly represent the transient activation induced by DAG. Furthermore, chronic exposure to these compounds downregulates PKCs, which further complicates data interpretation.

According to the first report in this field from 1977 by Pacifici and Holtzer, treatment of chondroblasts with PMA resulted in a rapid morphological change; *i.e.* chondroblasts have lost their polygonal morphology and at the same time ceased to synthesise sulphated ECM components [28]. They also reported that the observed negative effect was reversible if cells were exposed to PMA for up to 72 h only. However, longer-term treatment with PMA induced irreversible dedifferentiation [28]. Several years later, these initial results have been confirmed by another group who also observed that treatment with PMA reversibly inhibited chondrogenesis and influenced synthesis of ECM components [29]. Furthermore, PMA-treated limb bud-derived mesenchymal cells retained a flattened, fibroblast-like morphology and failed to accumulate type II collagen and other cartilage ECM components [30]. In view of the pronounced inhibitory effect of PMA on chondrogenic differentiation *in vitro*, these preliminary results provided indirect evidence that PKCs might play a positive role in chondroblast differentiation and function.

The results of Garrison and co-workers shed more light on how PMA exerted its effects on chondrogenesis [31]. They compared the effects of PMA to that of bryostatin I, a compound that also activates PKC and binds competitively to the putative 'PMA receptor', without influencing cell differentiation. According to their results, treatment of 4-day-old chick limb bud-derived micromass cultures with PMA markedly reduced cartilage formation; however, co-administration of bryostatin I and PMA at equimolar concentrations prevented the inhibitory effect of PMA on chondrogenesis, which has led to the conclusion that activation and/or downregulation of PKC alone cannot account for the effects of PMA. Nevertheless, by altering the time of PMA exposure they could establish the time frame and mechanism of its action; they demonstrated that PMA inhibited chondrocyte phenotypic expression, rather than

cell commitment, as early exposure to PMA (during chondrocytic commitment, *i.e.* in the first 48 h) had little inhibitory effect, whereas post-commitment exposure (during 0–96 h of culturing) caused a strong inhibition of matrix synthesis [31].

The findings of Biddulph and Dozier provided further evidence on the involvement of pathways triggered by phorbol esters in chondrogenic differentiation [32]. In that study, the effects of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) were investigated on chondrogenesis in high density cultures (HDC) established from chick limb bud-derived mesenchymal cells and also found that TPA completely blocked chondrogenesis when administered during the first 4 culturing days. Furthermore, they also measured prostaglandin E_2 (PGE_2) and cAMP concentrations and established that both parameters were altered by TPA treatment relative to untreated control cultures. In TPA-treated HDC both PGE_2 and cAMP levels increased progressively during the 6-day-long culturing period in contrast to an initial increase followed by a steady decline in untreated colonies [32]. The authors concluded that PGE_2 and cAMP-independent regulatory pathway(s) might be present in differentiating chondroprogenitor cells. The same results were published by a different group in 1990 [33]; moreover, they confirmed the chondro-inhibitory effect of TPA by proteoglycan staining procedures as well as RNA hybridisation to cDNA probes specific for cartilage ECM components. Furthermore, the effect of TPA was found to depend on initial plating density; at high density, TPA inhibited cell proliferation, whereas at low density, cell proliferation was stimulated by TPA. Once again, these results showed that the effects of phorbol esters on chondrogenesis are largely indirect; they possibly act by modulating cell proliferation [33].

In 1994, Ferrari and co-workers continued to study the effects of phorbol esters on chondrogenesis and reported that under the effect of TPA limb bud-derived mesenchymal cells exhibited dysregulated expression of the homeobox gene *Msx1*, implicated to suppress chondrogenic differentiation and maintenance of undifferentiated condition of chondroprogenitors [34]; furthermore, the formation of extensive gap junctions that normally occurs at the onset of chondrogenesis also failed to occur [35]. From these results the authors concluded that the chondro-suppressive effect of phorbol esters might stem from their ability to modulate the expression of key regulatory genes, as well as to interfere with the intercellular communication involved in the formation of differentiated chondrocytes.

4.2. Implications of PKC activity for chondrogenesis

It was not until 1988 that an active regulatory role for PKCs has been implicated in mediating insulin-like growth factor 1 (IFG-1) induced metabolic and mitogenic effects in isolated articular chondrocytes [36]. In that paper, the authors described that the effects of IGF-1, *i.e.* it enhanced sulphate and ^{3}H-thymidine incorporation,

could be mimicked by the phorbol ester 12,13-dibutyrate (PDBU), and that both effects could be blocked by the PKC inhibitor compound H7 [1-5(isoquinolinesulphonyl)-2-methylpiperazine]. They also observed that the L-type Ca^{2+} channel blocker verapamil could prevent IGF-1 and PDBU-stimulated sulphate—but not ^{3}H-thymidine—incorporation, which has led to the conclusion that PKC might regulate proliferation *via* a calcium-independent mechanism. Furthermore, since the cAMP analogue N^6,O^2-dibutyryl-adenosine-3′,5′-cyclic monophosphoric acid had the opposite effects (*i.e.* it enhanced matrix synthesis but reduced proliferation rate), the authors concluded that there is a close association between the activities of PKC and PKA enzyme systems in the mitogenic and metabolic processes of chondrocytes [36].

Although the results gained using phorbol esters and inhibitors indirectly implicated PKC isoenzymes as positive regulators of chondrogenesis, owing to lack of PKC subtype expression data, these early observations were unable to deliver strong evidence concerning their precise role. The initial findings describing the effects of these compounds on chondrogenesis *in vitro* are summarised in Fig. 3.

4.3. Early studies to assess the role of PKCs during chondrogenesis

It was hypothesised that if the antichondrogenic effects of PMA on embryonic limb bud-derived mesenchyme were mediated through PKC overactivation and subsequent downregulation, inhibition of PKC activity by a more specific inhibitor would have similar effects on cartilage formation. *Staurosporine*, an alkaloid derived from the bacterium *Streptomyces staurosporeus*, is a prototypical ATP-competitive kinase inhibitor which acts by preventing ATP from binding to the kinase; therefore, it is an aspecific protein kinase inhibitor which preferentially inhibits PKC with an IC_{50} value of 2.7 nM [37]. In 1991, however, Kulyk observed opposing effects using staurosporine on micromass cultures established from mesenchymal cells in embryonic chicken wing bud-derived micromass cultures [38]. Quite intriguingly, he found that treatments with staurosporine exerted chondro-stimulatory effects; it not only brought about elevated cartilage matrix accumulation

and enhanced cartilage-specific gene expression (cartilage-specific proteoglycan and collagen type II) under high density culture conditions (2×10^7 cells/mL) but it also induced extensive chondrocyte differentiation even in low density cultures (2.5×10^6 cells/mL) where cartilage formation does not spontaneously occur [38]. Furthermore, treatment with staurosporine was able to rescue the inhibitory effect of PMA, which suggested that the observed effects might be mediated through PKC. However, the chondrogenesis-promoting effect of staurosporine on chondrogenesis could not be unequivocally attributed to PKC modulation in that study, owing to the promiscuity of the inhibitor (see below). In fact, Kulyk hypothesised that in the developing limb bud, the chondro-inhibitory effect of the ectodermal jacket on chondroprogenitor mesenchymal cells might be mediated *via* signalling that involves phospholipase-mediated membrane phospholipid hydrolysis, DAG release, and resultant PKC activation. This theory is still yet to be confirmed.

By continuing this line of research, Kulyk and Reichert used HD cultures of mesenchymal cells from frontonasal, maxillary, and mandibular processes, as well as hyoid arches of stage 24/25 chicken embryos [39]. They found that staurosporine treatment enhanced chondrogenesis also in frontonasal and mandibular process mesenchyme, which spontaneously developed numerous chondrogenic cell aggregates; furthermore, it brought about extensive cartilage nodule formation in maxillary process mesenchyme, which formed little cartilage matrix under control conditions. Moreover, the presence of staurosporine initiated chondrocyte differentiation in hyoid arch mesenchyme, which demonstrated no spontaneous cartilage formation in control cultures [39]. These results indicated that PKC might be a negative regulator of chondrogenesis in chicken micromass cultures established from either facial or limb primordia; alternatively, the observed effects could be attributed to the highly aspecific nature of staurosporine as a protein kinase inhibitor.

Apart from *in vitro* studies using primary cultures of mesenchymal cells derived from embryonic limb buds or facial primordia, Ganan and co-workers attempted to confirm the role of PKC during digit
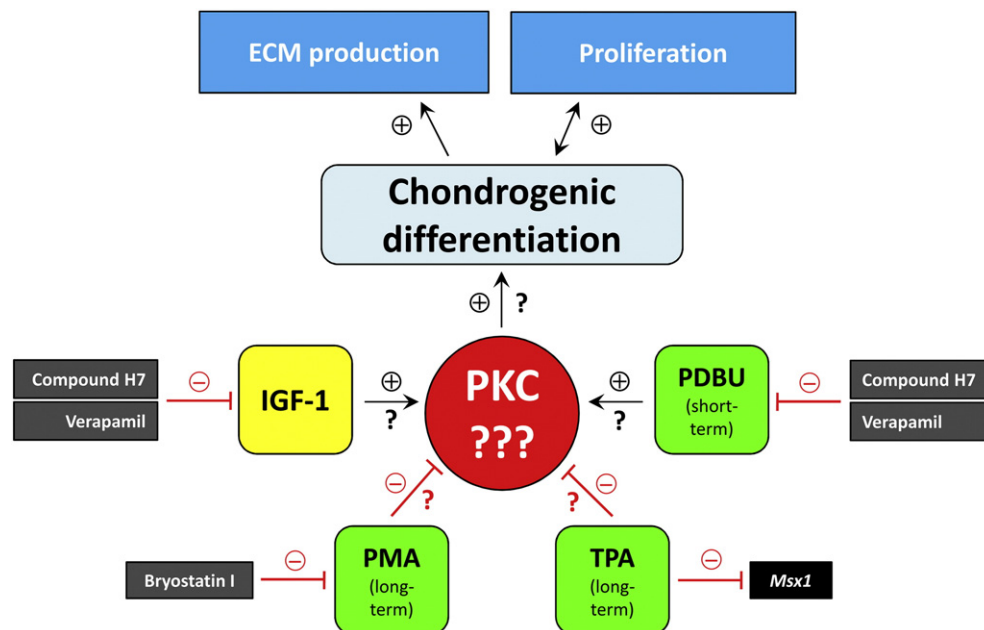
Fig. 3. Effects of phorbol esters and select compounds on chondrogenesis, implicating involvement of PKC signalling. Short-term exposure to PDBU enhanced chondrogenic differentiation and mimicked the chondrogenesis-promoting effects of the IGF-1 pathway, and this effect could be blocked by the PKC inhibitor compound H7 or the voltage gated Ca^{2+} channel blocker verapamil. Long-term exposure to PMA or TPA abrogated chondrogenesis; application of the competitive PKC inhibitor bryostatin I was able to compensate the effects of PMA. TPA altered the expression of *Msx1* that suppresses chondrogenic differentiation. These preliminary results implicated PKCs as positive regulators of chondrogenesis. Question marks indicate links that were either hypothetic or could not be confirmed at the time when these results were disseminated. Please see text for details and references. (ECM, extracellular matrix; IGF-1, insulin-like growth factor-1; PMA, phorbol-12-myristate-13-acetate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBU, 12,13-dibutyrate).

formation in chicken embryos *in vivo* [40]. Their initial observation was that at late stages of limb development, when the early patterning control mechanism has already ceased, the chick limb bud was able to form fully differentiated extra digits by removing the ectoderm over the interdigital spaces. They observed that microinjection of staurosporine resulted in the formation of fully differentiated extra digits, which appeared to be due to the induction of chondrogenesis following the inhibition of PKC. However, as mentioned before, all the above effects could also be attributed to the aspecific and promiscuous nature of staurosporine.

To resolve the apparent contradiction between results gained using either PMA or staurosporine, further attempts were made. By examining the temporal pattern of Sox9 expression in chicken embryonic wing bud-derived HDC, Kulyk and co-workers found that staurosporine rapidly upregulated *Sox9* mRNA, in advance of *Col2a1* transcript expression, which suggests that staurosporine affects chondrogenic differentiation by either directly or indirectly upregulating *Sox9* upstream of *Col2a1* [41]. Quite interestingly, they also found that PMA treatments caused a strong inhibition in *Col2a1* transcript levels, without seriously affecting *Sox9* mRNA expression, an observation suggesting that phorbol ester treatment inhibited *Col2a1* expression by a mechanism other than transcriptional control by *Sox9*. The authors suggested that by downregulating PKC, phorbol esters could have altered the phosphorylation state and activity of the Sox9 transcription factor, thus causing *Col2a1* downregulation.

To conclude, these preliminary observations using staurosporine as a compound to inhibit PKC are contradictory to what the previous set of experiments using phorbol esters suggested. However, as discussed later, these initial studies using compounds that are highly aspecific

could have led to inappropriate conclusions. Fig. 4 summarises the first results gained using staurosporine on chondrogenesis.

4.4. PKC isoform expression and activity during chondrogenesis

The previously mentioned studies either applied phorbol esters to activate and/or downregulate, or inhibitors such as staurosporine to block PKC activity; the authors of these papers made their conclusions solely by relying on the indirect pieces of evidence deduced from the effects of these compounds, without providing direct proof for the presence of PKC isoenzymes either at mRNA or protein levels, nor did they perform *de facto* PKC enzyme activity assays. It was not until 1993 that the latter flaw has been addressed by Sonn and Solursh, who assayed PKC activity for the first time in both cytosolic and particulate fractions of HD cultures established from stage 23/24 chick limb bud chondroprogenitor cells [42]. Initially, PKC activity was low, but during the course of chondrogenesis, cytosolic PKC activity significantly increased, while that of the particulate fraction increased only slightly. One of the most straightforward arguments of the determining role of PKC in the regulation of chondrogenesis was that PKC activity in cultures seeded at low density (5×10^6 cells/mL) where chondrogenesis is reduced was as low as that in 1-day-old cultures of cells plated at high density (2×10^7 cells/mL), which favours chondrogenic differentiation. These enzyme activity measurements unequivocally demonstrated that PMA inhibited chondrogenesis by depressing PKC; moreover, they also found that in the embryonic limb bud-derived system, staurosporine promoted chondrogenesis without affecting PKC activity—in contrast to earlier findings in micromass cultures established from embryonic chicken facial or wing primordia [38,39]. Thus, the fact that staurosporine acts
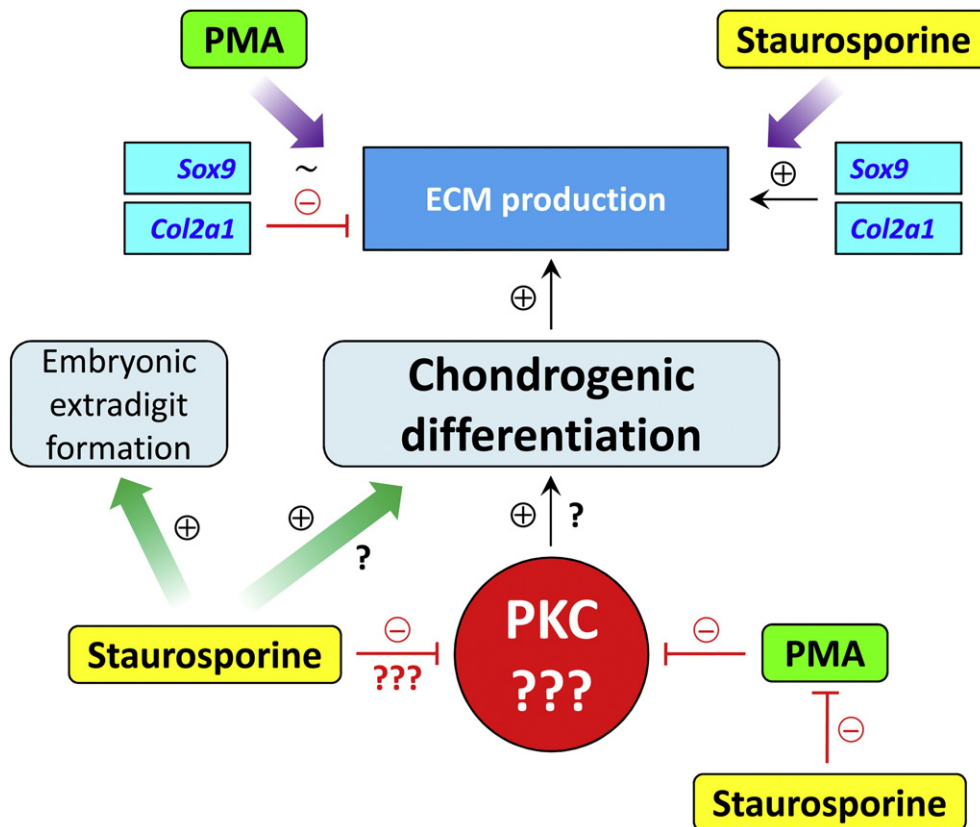
Fig. 4. Effects of staurosporine, an ATP-antagonist protein kinase inhibitor, on chondrogenesis. Treatment with staurosporine enhanced chondrogenic differentiation, upregulated *Sox9* and *Col2a1* mRNA expressions, and prevented the chondro-inhibitory effect of PMA. Furthermore, staurosporine induced extra digit formation in chicken embryos. However, most of these effects were later proved to be PKC-independent. Question marks indicate links that were either hypothetic or could not be confirmed at the time when data were disseminated. For further details and references, please see text. (ECM, extracellular matrix).

through a PKC-independent pathway to enhance *in vitro* chondrogenesis has been unequivocally confirmed.

Once *de facto* PKC enzyme activity measurements in chondrifying micromass cultures have been disseminated [42], the quest for identifying PKC isoenzymes expressed in cartilage at various stages of development has started. By using a pan-PKC antiserum recognising the catalytic domain of the enzyme, Bareggi and co-workers were the first to report that PKC was markedly expressed in chondrocytes in foetal mouse thoracic vertebrae [43]. In particular, chondrocytes were found to heavily express PKCβ, but they were also weakly stained by the anti-PKCε antiserum. Based on the immunohistochemical staining reactions, the authors concluded that PKCβ might play an important role in cartilage formation of the mouse axial skeleton. They then continued their experiments and performed an immunohistochemical analysis on the vertebral column of human embryos by using polyclonal antibodies to study the presence and distribution of nine PKC isoenzymes [44]. They investigated a key developmental period (developmental week 8), when the majority of chondrogenic differentiation processes occur during human embryonic development. Based on their results, the authors speculated that the appearance, localisation and activation of various PKC isoforms in chondrocytes might depend on the actual stage of chondrogenesis and that these enzymes may be key regulators of the process.

Shortly after these initial studies, Choi and co-workers examined the expression pattern of PKC isoenzymes in micromass cultures derived from the distal limb buds of chicken embryos with isoenzyme-specific anti-PKC antibodies, which gave a head start for further research in this field [45]. This group was the first to demonstrate that PKCα and PKCγ (but not PKCβ) of the conventional PKCs; PKCε (but not PKCδ) of the novel PKCs; as well as PKCλ/ι and PKCζ of the atypical PKCs were expressed throughout the 5-day-long culturing period. It is of note that they were unable to identify PKCβ in that system, and in this respect their data contradict to those of Bareggi and co-workers who reported that PKCβ was the PKC subtype with the strongest expression in developing mouse vertebrae [43]. Furthermore, since chondrogenesis was completely abrogated by applying specific PKC inhibitors such as bisindolylmaleimide or calphosptin, they confirmed earlier results concerning the determining role of PKCs in the regulation of cartilage formation. They could also make clear distinction between the involvement of different PKC subtypes; as chronic exposure to PMA considerably lowered the protein expression of cPKC and nPKC (but not aPKC) isoenzymes, they concluded that these subtypes were required for chondrogenesis to properly occur. By downregulating the expression of these PKCs with PMA at various stages of differentiation, the strongest inhibition of chondrogenesis was detected when PMA was applied for the first 24 h; furthermore, loss of PKC activity exerted a negative effect on both proliferation and cartilage ECM synthesis. These data indicate that PKCs are important regulators of the early stages of chondrogenesis.

In another report, Yang and co-workers established that among the PKC isoforms expressed in chicken limb bud-derived HDC at the protein level, expressions of PKCα and PKCε in the particulate membrane fraction were increased during *in vitro* chondrogenic differentiation, suggesting a determining role of these isoenzymes [46]. In support of this theory, by selectively blocking or downregulating PKCα, chondrogenesis could be inhibited; furthermore, there was a close correlation between the degree of chondrogenesis and the expression levels of PKCα, but not other PKC isoforms, which indicates that PKCα is a quintessential regulator of chondrogenesis [46]. Taken together, by comparing data derived from enzyme activity assays which suggested that PKC activity was initially low and then gradually increased as chondrogenesis progressed [42] with data from protein expression patterns showing that levels of PKCα, PKCγ and PKCε were initially low and then gradually increased with chondrogenesis, there is a perfect correlation between the two independent approaches.

In summary, the above results indicate that the expression and activation of PKCα act as an important induction signal for chondrogenic

differentiation of mesenchymal cells under micromass conditions: PKCα expression was low in undifferentiated progenitor cells but dramatically increased before chondrogenesis commenced in both cytosolic and particulate membrane fractions. The most important argument in supporting the central role of PKCα is that its inhibition was sufficient to block chondrocyte differentiation.

In a good correlation with the results of Choi and co-workers, Grill and colleagues verified the expressions of three PKC isoenzymes (PKCα, PKCε and PKCζ) in primary chondrocytes isolated from the tibiae of 6-day old chicken embryos [47]. In cells cultured for 20 days (*i.e.* they retained their chondrocyte phenotype) PKCε was the most prominently expressed isoform, whereas PKCα exhibited the least intense signals. To the contrary, once cells have undergone further differentiation towards the osteoblastic phenotype, PKCα was the most prominently expressed isoform, and a marked decrease of PKCε expression was detected. In terms of PKCζ expression no apparent differences have been detected between the two stages studied. Based on these findings, the authors concluded that these PKC isoenzymes may play roles at different levels of chondrocyte differentiation (*i.e.* mature *vs.* hypertrophic chondrocytes).

The same research team performed further immunocytochemical analyses on the expression of various PKC isoenzymes (*i.e.* PKCα, PKCδ, PKCε and PKCζ) in primary adherent cultures of chick chondrocytes derived from 6-day-old tibiae. They demonstrated that PKCζ exhibited a much stronger immunopositivity as compared to PKCα; furthermore, PKCδ and PKCε were even less expressed at the cell culture stage investigated [48]. Based on signal strength, the authors suggested that a major role could be played by PKCα and PKCζ in this particular phase of chondrogenesis. In another study when they monitored the expressions of PKC isoenzymes at various differentiation times (*i.e.* 48, 55, 62 and 69 days after cell collection from the tibiae of 6-day-old chick embryos), they reported that PKCζ was the isoenzyme that exhibited the strongest immunocytochemical expression in chondrocyte cultures at all ages, whereas PKCε has always proved to be the weakest as compared to the other PKC isoforms. As opposed to PKCε and PKCζ, no obvious differences in the expression patterns of PKCα and PKCδ could be observed at any time point [49]. The authors thus concluded that all PKC isoenzymes studied (*i.e.* PKCα, PKCδ, PKCε and PKCζ) may play peculiar roles in the differentiation process of chicken tibia-derived chondrocytes towards the osteoblastic phenotype.

In whole cell lysates of cultured primary human articular chondrocytes and C-20/A4 cells, Lee and colleagues investigated the expression of PKC isoenzymes using Western blotting and revealed that primary chondrocytes expressed PKC subtypes α, γ, δ, ι and λ, whereas PKCα, ι, λ and ε were identified in C-20/A4 cells [50]. Furthermore, they also described a role of PKCα in mediating chondrocyte mechanotransduction; following mechanical stimulation at 0.33 Hz, chondrocytes exhibited a rapid, integrin-dependent translocation of PKCα to the cell membrane, implicating an important role for PKC and integrin-mediated signalling.

LaVallie and colleagues also demonstrated the presence of multiple PKC subtypes in human chondrocytes [51]. However, in contrast to the previous investigators in this field, they used a different approach; they applied Affymetrix GeneChip® arrays to specifically identify mRNA transcripts of PKC family members. Probe sets for nine PKC isoforms were represented on the array as follows: α, β, γ, δ, ε, θ, η, ι, and ζ; of these, only four PKC isoforms, *i.e.* PKCδ, PKCι, PKCθ, and PKCζ were judged to be present in human mature chondrocytes by virtue of their hybridisation intensity levels. Moreover, of these four PKC isoforms, only PKCδ and PKCζ exceeded an empirically determined signal intensity threshold allowing reliable quantitation of their transcript levels on the gene chips [51]. The fact that PKCα mRNA transcript levels were not considered to be heavily expressed could stem from the different experimental setups applied in that study. In agreement with these data, our laboratory was the first to show PKCδ mRNA and protein expression during *in vitro* chondrogenesis in chicken embryonic limb

Table 1

Chronological order of publications describing PKC isoenzyme expression and function during chondrogenesis and/or in chondrocytes derived from various model systems using various experimental approaches.

	Organism and model system	PKC isoenzyme(s)	Comment	Reference
1.	HDC from the distal limb buds of chicken embryos	Overall PKC activity	Measured PKC activity in cytosolic and particulate fractions for the first time. PMA inhibited chondrogenesis by reducing PKC activity; staurosporine had PKC-independent effects	[42]
2.	Foetal mouse thoracic vertebrae	PKCβ, PKCε	Implication of PKCβ as main isoform during cartilage formation of the mouse axial skeleton by immunohistochemical approach	[43]
3.	Vertebral column of human embryos	Various PKC isoforms	Stage-dependent appearance of various PKC isoforms during cartilage formation by immunohistochemical approach	[44]
4.	HDC from the distal limb buds of chicken embryos	cPKC: PKCα, PKCγ; nPKC: PKCε; aPKC: PKCλ/ι, PKCζ	Confirmed expression of specific PKC isoforms using immunoblots. Inhibitors (bisindolylmaleimide or calphosptin) abrogated chondrogenesis. Confirmed that PMA downregulates PKC.	[45]
5.	HDC from the distal limb buds of chicken embryos	PKCα, PKCε	Increased expression of PKCα and PKCε in the particulate membrane fraction during chondrogenesis; by selectively blocking PKCα, chondrogenesis was inhibited; close correlation between the degree of chondrogenesis and PKCα expression levels—PKCα is a quintessential regulator of chondrogenesis	[46]
6.	Chondrocytes isolated from the tibiae of 6-day old chicken embryos	PKCα, PKCε, PKCζ	PKCε prominently expressed and PKCα showed less intense signals in chondrocytes; opposite pattern in osteoblastic cells; no difference in PKCζ expression	[47]
7.	Chondrocytes isolated from the tibiae of 6-day old chicken embryos	PKCα, PKCδ, PKCε, PKCζ	PKCζ exhibited a much stronger immunopositivity than PKCα; PKCδ and PKCε were even less expressed. PKCα and PKCζ are key regulators	[48]
8.	Primary cultured human articular chondrocytes and C-20/A4 cells	PKCα, PKCγ, PKCδ, PKCι and PKCλ in primary cells; PKCα, PKCι, PKCλ and PKCε in C-20/A4	Western blotting revealed that primary chondrocytes expressed PKC subtypes α, γ, δ, ι and λ, whereas only PKCα, ι, λ and ε were identified in C-20/A4 cells. Role of PKCα in mechanotransduction pathways.	[50]
9.	Chondrocytes isolated from the tibiae of 6-day old chicken embryos; time series	PKCα, PKCδ, PKCε, PKCζ	PKCζ exhibited strongest immunocytochemical expression at all time points; PKCε was always the weakest	[49]
10.	Mature human chondrocytes	PKCδ, PKCι, PKCθ, PKCζ	Affymetrix GeneChip® array analysis; only PKCδ and PKCζ exhibited strong expression levels	[51]
11.	HDC from the distal limb buds of chicken embryos	PKCδ	PKCδ mRNA and protein expressions; PKCδ has chondrogenesis-promoting effects	[52]

bud-derived micromass cultures and described it as an important positive regulator of chondrogenic differentiation of mesenchymal cells [52]. Various PKC isoenzymes described during chondrogenesis and/or in mature chondrocytes by different laboratories are shown in Table 1.

Once the presence and central role of many PKC isoenzymes has been confirmed at both mRNA and protein levels, as well as PKC activity has also been detected during chondrogenesis, attempts to understand their precise role in mediating signalling processes to drive *in vitro* chondrogenesis have been initiated. In the following section of this review article, we are providing an overview of what processes and signalling pathways are implicated to be involved in PKC signalling during chondrogenesis.

5. PKCs regulate the chondrocyte phenotype *via* the actin cytoskeleton

The actin cytoskeleton serves numerous vital functions in any cell type. Besides providing cells with structural and mechanical stability, it also serves as a major organiser for signalling pathways that regulate adhesion and intercellular communication. The cytoskeletal architecture can undergo rapid changes in conformation and arrangement in response to extracellular stimuli, which can lead to metabolic activation, differentiation, proliferation, or apoptosis. Healthy chondrocytes are characterised by a faint microfibrillary actin cytoskeleton; in contrast, dedifferentiating chondrocytes with fibroblastic morphology lack this well organised network and possess thick actin cables (stress fibres) along with altered ECM and collagen synthesis [53]. A possible role for the actin cytoskeleton in chondrocyte differentiation was suggested by the initial observations on the ability of spherical chondrocytes cultured in agarose gels to maintain chondrogenic phenotype and conversely, to lose it when cultured in monolayers [54,55].

Staurosporine as well as other PKC inhibitors such as PMA or compound H7 have long been shown to rapidly disorganise the microfilament cytoskeletal network and consequently induce changes in cell shape and adhesion [56]. Given that a transition from a fibroblastic shape to a round chondrocyte-like phenotype, through actin reorganisation, is a prerequisite to chondrogenesis [57], Borge and co-workers tested the hypothesis

whether reorganisation of the actin cytoskeleton restored the differentiated phenotype of serially passaged undifferentiated chondrocytes [58]. They found that treatment with staurosporine restored the synthesis of collagen type II and sulphated ECM components and at the same time suppressed collagen type I and III production. The authors concluded that the capacity of staurosporin to reinduce the expression of the differentiated phenotype in chondrocytes could primarily be attributed to the reorganisation of the actin microfilaments, rather than a consequence of PKC inhibition. In another study by Hoben and colleagues staurosporine was applied as an actin-modifying agent to increase the gene expression and synthesis of major fibrocartilage ECM components in a monolayer culture of fibrochondrocytes and established that staurosporine could be used to enhance matrix production and reduce contraction in tissue-engineered fibrocartilage constructs, without implicating the involvement of PKC subtypes [59].

Nevertheless, Lim and co-workers provided direct evidence that disruption of the actin cytoskeleton by cytochalasin D enhances *in vitro* chondrogenesis by activating PKCα and by inhibiting the ERK-1 signalling pathway [60]. Using embryonic chicken wing bud-derived mesenchymal cells cultured in subconfluent clonal cell density (6×10^5 cells/mL), both cytochalasin D and latrunculin B induced chondrogenesis *via* increased expression and activation of PKCα. In accordance with the above findings, extensive stress fibre network was observed in chondrogenic cells seeded at this low density, which is non-permissive for chondrogenesis to take place. However, disorganisation of the actin cytoskeleton by cytochalasin D failed to initiate chondrogenesis when PKCα was concurrently inhibited with Gö6976, a rather specific inhibitor of PKCα, or depleted by chronic exposure to PMA. Since the expression levels of other PKC isoforms such as PKCε and PKCλ/ι did not significantly change in the presence of cytochalasin D, these data clearly indicated that signalling through PKCα was essential for chondrogenic differentiation of limb bud-derived mesenchymal cells at clonal cell densities.

The link between actin cytoskeleton and PKC signalling in chondrocytes has been further studied by Kim and co-workers. It has long been known that exposure to nitric oxide (NO) initiates dedifferentiation and/or apoptosis in mature chondrocytes *via* signalling that involves the MAPK cascade and by inhibiting PKCα and PKCζ [61]. By

using reagents that either inhibit actin polymerisation and disrupt existing actin filaments, or induce actin polymerisation and stabilise actin in a polymeric form, Kim and colleagues could unequivocally prove that the actin cytoskeletal architecture is a critical regulator of articular chondrocyte function [62]. They found that disruption of the actin cytoskeleton structure prevents NO-induced dedifferentiation by eliminating the NO-evoked inhibition of PKCα and PKCζ.

In 2007, Nurminsky and co-workers supplied further evidence on the interdependence of cytoskeletal organisation and chondrogenic gene expression [63]. They found that the expression of the gelsolin-like actin-binding protein adseverin (also known as scinderin) is dramatically upregulated during chondrocyte maturation in a PKC dependent manner in chicken chondrocytes. Treatments with PKC inhibitor bisindolylmaleimide I (GF109203X) caused dedifferentiation of chondrocytes: cells acquired fibroblastic morphology along with complete abrogation of Ihh and collagen type X expression. Adseverin expression was similarly reduced in bisindolylmaleimide-treated chondrocytes, which implicated that adseverin upregulation is specifically linked to the chondrocyte maturation programme and is regulated by the PKC pathway [63]. Although the latter study was performed on terminally differentiating chondrocytes, it is plausible that the same or at least similar molecular mechanism may control changes in cell shape during primary chondrocyte differentiation.

The recently published paper by Rottmar and his group also came to the same conclusions: both staurosporine and cytochalasin D augmented chondrogenic gene expression in dedifferentiated fibroblastic bovine articular chondrocytes, and the PKC pathway was a positive regulator of this phenomenon [64].

Based on the above findings, the following model for PKC-induced actin cytoskeleton reorganisation can be proposed (Fig. 5). During chondrogenesis, PKC activity increases, which induces changes in the expression patterns of various factors including adseverin that causes reorganisation of the actin cytoskeleton, thereby promoting changes in cell shape from a fibroblastic to a rounded chondrocyte-like phenotype.

6. PKC exerts its chondrogenesis-promoting effect *via* the ERK–MAPK pathway

Although PKCs have long been known to positively regulate *in vitro* chondrogenesis, the mechanisms through which they exert their functions are yet to be clarified. Since MAP-kinases are central regulators of chondrogenic differentiation [65], it is not surprising that among other signalling systems, PKCs are also upstream modulators of this pathway. According to what has been described earlier, the MAPK family comprises three major cytoplasmic cascades: ERK1/2, p38, and JNK pathways. Given the prominent role of the ERK1/2 pathway in the regulation of chondrogenesis, it is quite evident that the majority of research aiming at establishing a link between PKC and MAPK signalling has been carried out with the ERK1/2 pathway in focus.

6.1. PKC mediates chondrogenesis via the ERK1/2 pathway

The first link between PKC and ERK1/2 pathways has been established by Scherle and co-workers in 1997. They found that a 20-min-long treatment with the potent PKC activator PDBU evoked a more than 10-fold stimulation of ERK1/2 activity in isolated rabbit articular chondrocytes, without affecting p38 and JNK activities [66]. In 1998, Chang and co-workers have confirmed these results, demonstrating that PKC regulates chondrogenesis by modulating N-cadherin expression *via* ERK-1 activity in chicken wing bud-derived
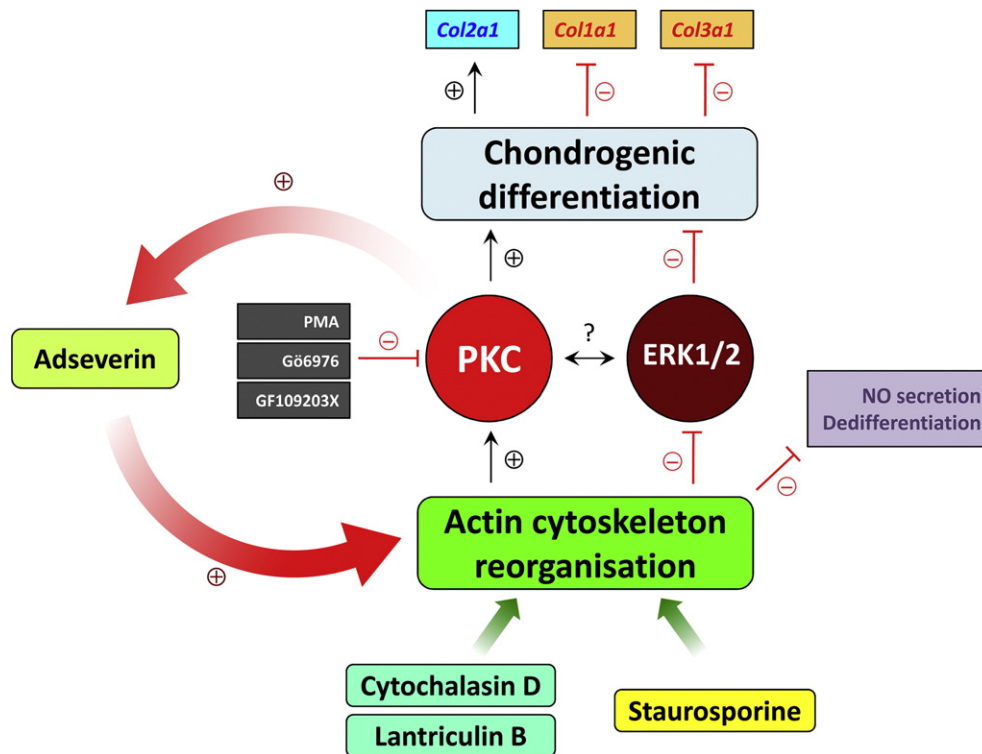
Fig. 5. Proposed model for PKC-induced actin cytoskeleton reorganisation during chondrogenesis. Compounds that induce reorganisation of the actin cytoskeleton (staurosporine, cytochalasin D, lantriculin B) enhance PKC activity and at the same time inhibit ERK1/2; both pathways promote chondrogenic differentiation and induce cartilage-specific gene expression (*i.e.* upregulate *Col2a1*, and downregulate *Col1a1* and *Col3a1*). Nitric oxide (NO)-induced dedifferentiation of the chondrocyte phenotype is also blocked. Enhanced PKC activity that is indispensable to chondrogenesis induces changes in the expression patterns of various factors including adseverin that causes reorganisation of the actin cytoskeleton, thereby promoting changes in cell shape from a fibroblastoid to a rounded chondrocyte-like phenotype. Various PKC inhibitors (PMA, Gö6976, GF109203X) exert negative effects on this pathway. At this level, a putative link between PKC and ERK1/2 signalling has not been established. Please see text for further explanation and references. Note that in the chicken limb bud-derived HD experimental model applied in these initial studies, activation of ERK1/2 negatively regulates chondrogenesis.

HDC [67]. This conclusion was derived from the following main arguments:

(1) Inhibition of PKC enzyme activity resulted in the activation of ERK-1, and at the same time chondrogenesis was blocked.
(2) Inhibition of ERK-1 with PD98059 (2-[amino-3-methoxyphenyl]-4H-1-benzopyran-4-one) augmented chondrogenesis; furthermore, it was able to rescue decreased rate of chondrogenesis brought about by blockade of PKC activity.
(3) ERK-1 inhibition did not affect expression and subcellular distribution of PKC isoforms in micromass cultures.
(4) Inhibition of PKC activity modulated the expression of cell adhesion molecules (N-cadherin, integrin) involved in precartilaginous condensation, which was reversed by the inhibition of ERK-1.

From these data, the authors concluded that ERK-1 modulates synthesis and/or activation of transcription factors that regulate the expression of cell adhesion molecules and ECM components *via* signalling that involves PKC.

The fact that PKC is acting upstream of ERK1/2 was reinforced by Oh and colleagues in 2000. They confirmed that the amounts and expression patterns as well as the distribution of PKC isoforms expressed in chondrifying micromass cultures between cytosolic and particulate membrane fractions were essentially the same in the absence or presence of the p38 inhibitor SB203580 (4-[4-fluorophenyl]-2-[4-pyridyl]-1H-imidazole) or the ERK1/2 inhibitor PD98059; conversely, inhibition of PKC with the PKCα and PKCβ-specific inhibitor Gö6976, or down-regulation of PKC with prolonged exposure of cells to PMA resulted in an increased level of P-ERK-1 and consequent inhibition of chondrogenesis. Moreover, in untreated control cultures, the decrease in ERK1/2 activities that favours chondrogenesis was highly correlated with the increased expression and activation of PKCα and PKCε isoforms [8].

As an attempt to identify an upstream activator of PKCα in differentiating chondroprogenitors, the molecular mechanism of the glucose effect on the regulation of chondrogenesis has been studied in detail by Han and colleagues. They observed that exposure of micromass cultures derived from chick wing bud mesenchymal cells to high concentrations of glucose (30 mM or higher) significantly stimulated chondrogenesis without affecting cell proliferation. Furthermore, their results implied that the observed effects could be attributed to PKCα; its levels and the membrane translocation were increased by glucose treatment, leading to a reduction of P-ERK1/2 levels, and at the same time enhancing p38 phosphorylation. Apart from changes in phosphorylation status of key signalling targets, glucose also modulated the expression patterns of cell adhesion molecules including fibronectin, integrin β1, and N-cadherin. The key argument in favour of their hypothesis was that these alterations in protein level of adhesion molecules and in the phosphorylation status of MAPKs could be blocked by inhibition of PKC using GF109203X, which has led to the conclusion that high doses of glucose induce the downregulation of ERK1/2 activity in a PKCα dependent manner and stimulate *in vitro* chondrogenesis *via* modulating the expression of key adhesion molecules [68].

The results of our laboratory suggest that the novel PKC isoform PKCδ also regulates chondrogenesis, at least partially, *via* the ERK1/2 pathway [52]. In this study, either the natural compound rottlerin was applied or transient gene silencing was performed using PKCdelta shRNA to inhibit PKCδ activity. Both rottlerin and PKCδ shRNA caused a severe reduction in cartilage formation, furthermore protein and phospho-protein levels of Sox9, the key transcription factor of chondrogenesis, were also significantly decreased. However, rottlerin lowered, while PKCδ gene silencing elevated the phosphorylation status of ERK1/2. Our data suggested that PKCδ stimulated chondrogenesis *via* influencing Sox9 and ERK1/2 phosphorylation, but the inhibition of cartilage formation in the rottlerin-treated HDC was probably partially PKCδ independent.

6.1.1. PKC–ERK1/2 signalling through the actin cytoskeleton

There are many other processes during chondrogenesis and/or in mature chondrocytes in which PKC-mediated MAPK signalling plays a central role. As mentioned earlier, disruption of the actin cytoskeleton by cytochalasin D induced chondrogenesis of mesenchymal cells even at low cellular densities *via* a PKCα dependent mechanism [60]. At the same time, treatment with cytochalasin D reduced ERK-1 phosphorylation, which is consistent with other observations suggesting that ERK activation is regulated by reorganisation of the actin cytoskeleton [69]. Since ERK-1 is a negative regulator of chondrogenesis in chick limb bud-derived mesenchymal cells [67], the authors assumed that the decrease in P-ERK-1 levels could at least partially be accounted for enhanced chondrogenesis. However, application of the ERK-1 inhibitor PD98059 failed to further enhance the induced chondrogenesis, which suggested that the ERK-1 pathway alone was insufficient for inducing chondrogenesis and other pathways were also involved.

As it was discussed earlier, Nurminsky and co-workers found a close interdependence of actin cytoskeletal organisation and chondrogenic gene expression in a PKC-dependent manner during terminal differentiation of hypertrophic chicken chondrocytes [63]. Moreover, since application of the MEK-inhibitor U0126 also resulted in similar inhibitory effects, involvement of the MEK–ERK1/2 pathway was also implicated. Thus, they concluded that activation of PKC signalling in non-hypertrophic chondrocytes induces adseverin expression in a MEK-dependent manner (see Fig. 5). Similar findings were reported also by Rottmar and colleagues who found that the p38-MAPK pathway positively regulated chondrogenesis, while the ERK-pathway was a negative regulator in staurosporine-induced re-differentiation [64].

Based on the results summarised in this section, the proposed mechanism of PKC-dependent regulation of chondrogenesis *via* the ERK1/2 MAPK pathway and modulation of cell adhesion molecules is shown in Fig. 6.

6.2. PKC, p38 MAPK and chondrogenesis

The increased expression and activity of PKC are required for downregulation of ERK-1, a negative regulator of *in vitro* chondrogenesis, which correlates well with the induction of chondrogenic differentiation of embryonic chondroprogenitor cells. In contrast to ERK-1, p38 activity is increased during chondrogenesis, and inhibition of p38 MAP kinase blocks chondrogenesis [8]. As mentioned above, Oh and co-workers provided important data that ERK1/2 is a downstream target of PKC signalling in chick limb bud-derived chondrifying micromass cultures; however, they also found that neither inhibition of classic PKC isoenzymes with Gö6976 nor downregulation with prolonged exposure to PMA affected p38 activity in these cultures. These results indicated that unlike ERK1/2, p38 was not linked to the PKC signalling pathway [8].

Later on, the same research group observed that the chondrogenesis-inhibitory effects of the immunosuppressant rapamycin were mediated *via* inhibition of PKCα activation (but not the expression) during nodule formation, without altering the phosphorylation status of ERK-1 [70]. In contrast, p38 kinase activity was found to be dramatically reduced when cells were exposed to rapamycin, with concurrent inhibition of chondrogenesis. However, as the inhibition or downregulation of PKCα did not affect p38 activity, the rapamycin-mediated regulation of PKCα signalling appeared to be independent of p38 MAP kinase signalling.

To further dissect the molecular mechanism of PKC-mediated MAPK signalling during chondrogenesis and to search for possible upstream regulators, Jin and co-workers studied the possible interrelation between TGF-β3 and Wnt-5a, two important modulators of chondrogenic differentiation of mesenchymal cells. They demonstrated that TGF-β3 stimulates *in vitro* chondrogenesis by upregulating Wnt-5a expression. Furthermore, the downstream targets of this pathway include PKCα and p38 as overexpression of Wnt-5a or treatment with exogenous TGF-β3 stimulated the activation of these two kinases, whereas
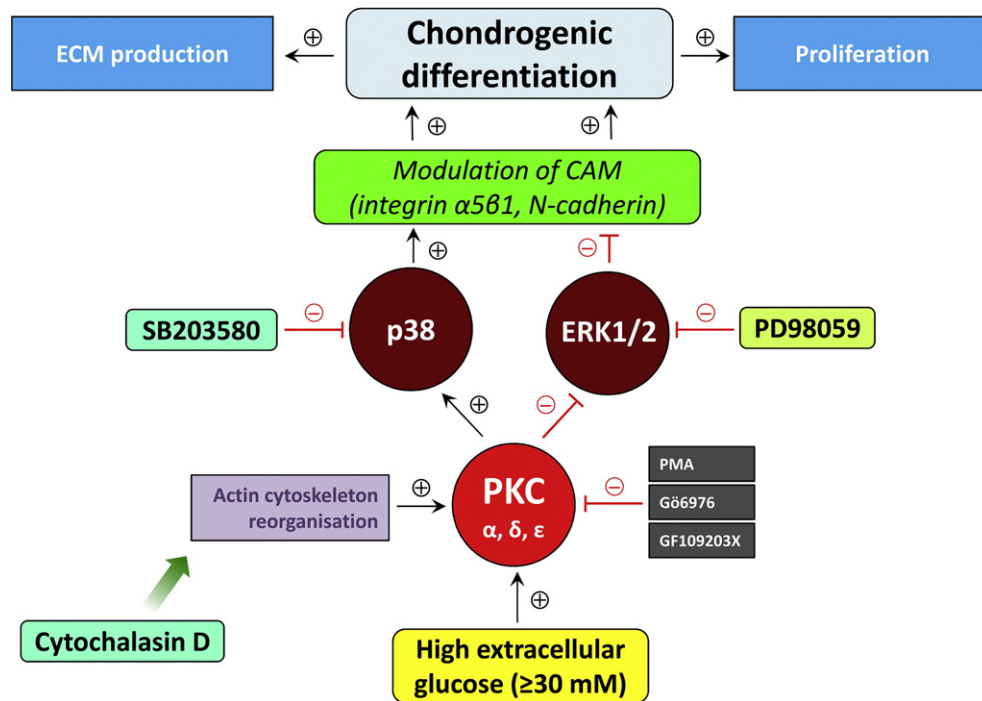
Fig. 6. Model to explain the role of PKC (primarily PKCα, PKCδ and PKCε) in mediating the effects of high extracellular glucose (≥30 mM) and reorganisation of the actin cytoskeleton. Based on the initial results, the hypothesis is that ERK1/2 modulates the expression of cell adhesion molecules (*i.e.* integrin, N-cadherin) and ECM components *via* signalling that involves PKC. For further details and references please see text. Note that in the chicken HDC applied in these studies, ERK1/2 is a negative regulator of chondrogenesis. (CAM, cell adhesion molecule; ECM, extracellular matrix; SB203580, p38 MAPK inhibitor; PD98059, ERK1/2 MAPK inhibitor).

inactivation of PKCα and p38 by their specific inhibitors (GF109203X, or by depletion of cytosolic Ca^{2+} with EGTA; or PD169316, respectively) completely abrogated chondrogenesis stimulated by both TGF-β3 and Wnt-5a. These results implicated that Wnt-5a mediates the chondro-stimulatory effect of TGF-β3 through upregulation of PKCα and p38 signalling [71].

6.2.1. PKC–p38 signalling through the actin cytoskeleton

As discussed earlier, Lim and co-workers have shown that disruption of the actin cytoskeleton with cytochalasin D in subconfluent chick limb bud-derived chondroprogenitor cells induced chondrogenic differentiation, mediated through increased expression and activity of PKCα [60]. Since it has long been known that disassembly of actin filaments has altered the phosphorylation status of both ERK1/2 and p38 [72], Lim and co-workers undertook to investigate the possible role of ERK1/2 and p38 in PKCα-regulated chondrogenesis of subconfluent mesenchymal cells induced by cytoskeleton disruption. They demonstrated that activation of p38 was necessary for cytochalasin D-induced chondrogenesis. Furthermore, inhibition or downregulation of PKC by the potent and selective PKC inhibitor GF109203X (bisindolylmaleimide I) or PMA, respectively, blocked cytochalasin D-induced p38 activation, whereas inhibition of p38 with SB203580 did not alter the expression and activity of PKC. These results imply that a PKC–p38 MAP kinase pathway regulates the induction of chondrogenesis by disruption of the cytoskeleton [73], which is an important difference compared to micromass culture-induced chondrogenesis when p38 is independent of PKC signalling [8]. Similar conclusions were drawn also from the observations of Rottmar and colleagues [64]. Fig. 7 shows a schematic pathway of the involvement of PKC and p38 MAPK signalling during chondrogenesis.

6.3. Chondrocyte de- and redifferentiation are regulated by PKC and MAPK signalling

Once the signalling mechanisms involving the PKCα-dependent ERK-1 pathway that governs chondrocyte differentiation have been

clearly elucidated, Yoon and colleagues undertook to decipher the functions of this signalling in maintaining the chondrocyte phenotype [74]. They used serially passaged monolayer cultures of chondrocytes derived from rabbit articular cartilage and studied their de- and redifferentiation in three-dimensional cultures. The fact that PKCα was required for the maintenance of differentiated chondrocyte phenotype was confirmed by the observation that its downregulation by prolonged treatment with PMA caused dedifferentiation. Interestingly, since treatment of chondrocytes with the PKC inhibitors GF109203X or Gö6976 did not cause dedifferentiation, the authors speculated that it is the expression, rather than the activity, of PKCα that was required for the maintenance of differentiated chondrocyte phenotype. At the same time, they also demonstrated that PKC and ERK1/2 independently regulated dedifferentiation of chondrocytes since PKCα overexpression did not block enhanced ERK phosphorylation required for the recovery of chondrocyte phenotype. In conclusion, these and earlier data suggest that chondrocyte differentiation in micromass cultures is mediated by PKC-dependent ERK1/2 regulation, while PKCα and ERK1/2 separately control chondrocyte dedifferentiation [74].

The relationship between PKC signalling and p38 MAPK has been further analysed by Lee and colleagues in 2007 who investigated the effects of chitosan on the redifferentiation of dedifferentiated chondrocytes obtained from a micromass culture system of chick wing bud mesenchymal cells. Mature chondrocytes were subjected to serial monolayer subculturing which yielded dedifferentiated cells. When the dedifferentiated chondrocytes were cultured on chitosan membranes they regained the phenotype of differentiated chondrocytes. They also observed that expression of PKC increased during chondrogenesis, decreased during dedifferentiation, and increased again during redifferentiation, implicating an important regulatory role of this pathway. This was further supported by the fact that PMA-treatment of dedifferentiated chondrocytes inhibited redifferentiation and downregulated PKC. In addition, the expression of p38 MAPK increased during redifferentiation and its inhibition suppressed redifferentiation. If we consider the fact that chondrocyte de- and redifferentiation also
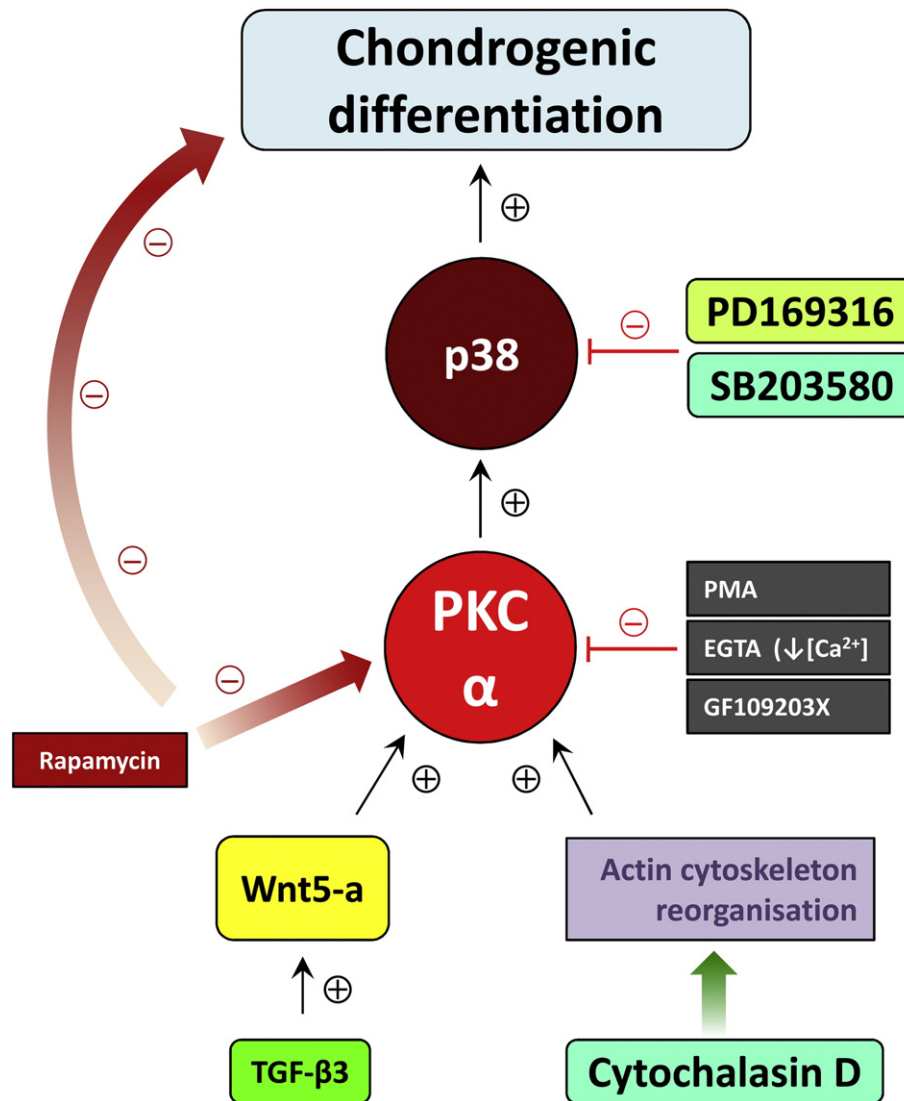
Fig. 7. PKC signalling is involved in activating the p38 MAPK pathway to enhance chondrogenesis. TGF-β3 stimulates chondrogenic differentiation through upregulation of Wnt5-a, which in turn activates the PKCα–p38 pathway. Disruption of the actin cytoskeleton by cytochalasin D can also activate the p38 MAPK pathway in a PKCα dependent manner. Note that the PKC–p38 MAPK pathway is reported to regulate the induction of chondrogenesis by disruption of the cytoskeleton, unlike micromass culture-induced chondrogenesis when p38 is independent of PKC signalling. Similarly, rapamycin suppressed chondrogenesis *via* a p38 independent mechanism. For more detailed explanation and relevant references, please see text. (SB203580 and PD169316, p38 MAPK inhibitors).

involve changes in cell morphology, accompanied by profound reorganisation of the actin cytoskeleton, these findings indicate that PKC and p38 activities, along with cytoskeletal reorganisation, are essential for chondrocyte redifferentiation [75]. Fig. 8 depicts the PKC-dependent regulation of chondrocyte differentiation, dedifferentiation and redifferentiation processes.

6.4. PKC mediates the effects of IGF-1 and EGF during chondrogenesis

IGF-1 is known to be expressed in developing cartilage within the condensing region of the embryonic limb bud as well as in mature cartilage and synovial fluid, where it regulates chondrogenesis and anabolism of cartilage ECM molecules. As described above, Taylor and colleagues suggested an active regulatory role for PKCs in mediating IGF-1 induced metabolic and mitogenic effects in isolated articular chondrocytes [36] (see Fig. 3). However, at that time, they did not aim to provide a detailed analysis of the signalling pathways involved in mediating the effects of IGF-1. Several years later Oh and Chun published a paper on the signalling mechanisms leading to IGF-1 action

[76]. According to their results, IGF-1 induces chondrogenic differentiation of chick limb bud-derived mesenchymal cells by activating PI-3K, which in turn leads to the subsequent activation of PKCα and p38, and at the same time inhibition of ERK1/2. Their results indicated that although the expression and activation of PKCα were required for chondrogenesis to take place, ectopic expression of PKCα alone was insufficient to activate p38 and subsequently to induce chondrogenesis in the absence of IGF-1 or serum.

During embryonic limb development, epithelial cells in the apical ectodermal ridge (AER) keep the underlying mesenchymal cells in state of mitotic proliferation, which prevents differentiation, by secreting signalling molecules such as the epidermal growth factor (EGF). When Yoon and co-workers scrutinised the molecular mechanism by which EGF inhibits chondrogenesis they found that exogenous EGF treatment inhibited the expression and activation of PKCα, and at the same time activated ERK-1 and inhibited p38, all of which has led to inhibition of chondrogenesis. Furthermore, stimulation of the EGF receptor blocked precartilage condensation and altered the expression of cell adhesion molecules such as N-cadherin and integrin α5β1; all
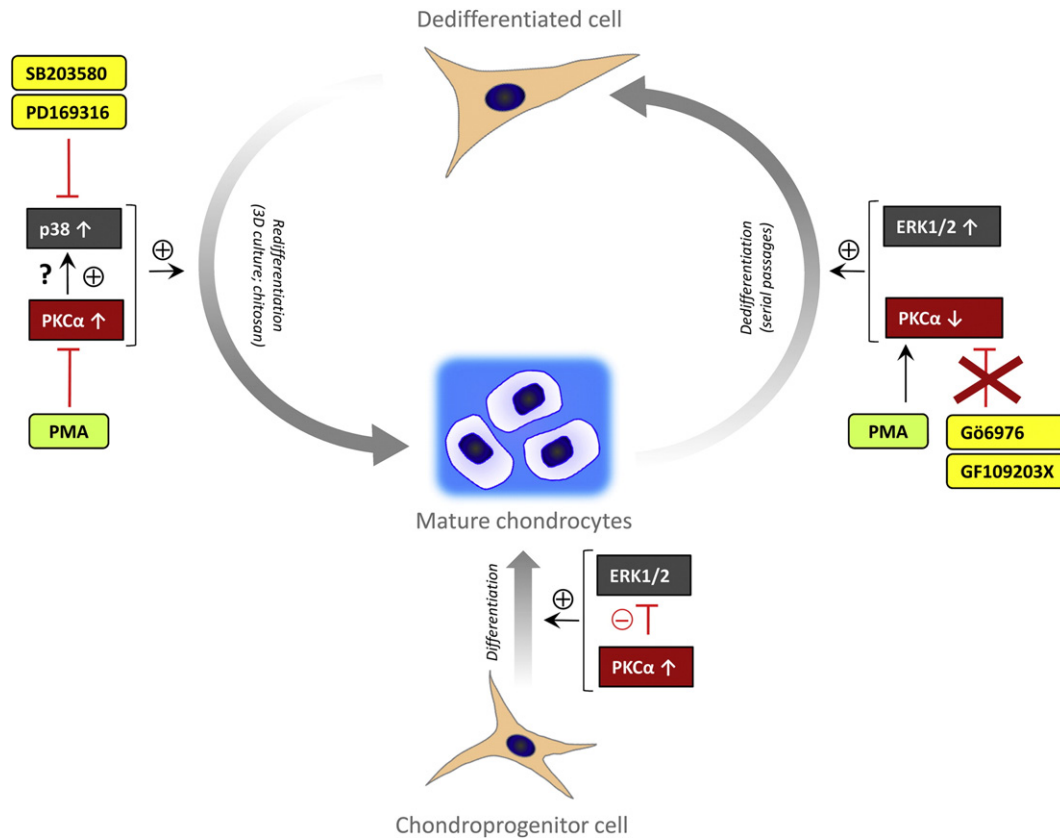
Fig. 8. Schematic diagram depicting the suggested role of PKCα, ERK1/2 and p38 MAPK in differentiation, dedifferentiation, and redifferentiation of chondrocytes. PKCα acts as a positive regulator during differentiation of chondroprogenitor cells to chondrocytes and redifferentiation of fibroblastoid cells to chondrocytes, whereas a decrease in PKCα expression causes dedifferentiation of mature chondrocytes. In contrast, activation of ERK1/2 causes dedifferentiation and blocks differentiation and redifferentiation. p38 has been implicated as a positive regulator of redifferentiation. PKC exerts its effects on differentiation by inhibiting ERK1/2 signalling, whereas PKC and ERK1/2 independently regulate dedifferentiation of chondrocytes. The question mark indicates a hypothetic link between PKC and p38. See text for further details.

these effects could be blocked by the application of the EGF receptor inhibitor AG1478 [77]. Since selective inhibition of PKCα was sufficient to inhibit *in vitro* chondrogenesis, it can be hypothesised that the EGF-induced modulation of PKCα-dependent signalling is the key factor of the inhibition of chondrogenesis by EGF. The schematic drawing in Fig. 9 shows how IGF-1 and EGF exert their distinct functions in a PKC-dependent manner during chondrogenesis.

6.5. Crosstalk between JNK and PKC pathways

The third MAPK signalling pathway, the c-Jun N-terminal kinase (JNK)—also known as the stress-activated protein kinase (SAPK)—pathway is strongly activated by a multitude of noxious stimulants such as heat shock, ionising radiation, oxidant stress, and mechanical shear. However, in contrast to ERK1/2, the JNK pathway is not strongly activated by growth factors and mitogens in the majority of cell types studied [65]. Moreover, relative to the ERK1/2 and p38 pathways, substantially less research has been carried out in searching for the regulatory roles of JNK signalling during chondrogenesis. As previously described, Oh and colleagues demonstrated that unlike ERK and p38, the phosphorylation of JNK was not detectable during chondrogenesis in micromass cultures of embryonic chick wing bud mesenchyme [8]. In accordance with these observations, treatment with the JNK inhibitor SP600125 did not influence cartilage matrix formation in micromass cultures prepared from chick embryonic mesenchyme [65]. In contrast, Hwang and co-workers described that Wnt-3a caused the expression of c-Jun and its phosphorylation by JNK, resulting in activation of AP-1, which in turn suppressed the expression of Sox9 [9].

In chondrocytes, the effects of CCN2/connective tissue growth factor (CCN2/CTGF) are known to promote both proliferation and differentiation of chondrocytes *via* the ERK1/2, p38 and JNK MAPK pathways [78]. In terms of upstream kinases of this pathway, the involvement of PKCα, PKCε and PKCζ, as well as PI-3K and PKA has been implicated to promote the maturation, proliferation and terminal differentiation of chondrocytes. Although application of the PKC inhibitor calphostin C repressed all of the effects of CCN2 and it also exerted inhibitory effects on the activation of p38 and ERK1/2, it had no effect on JNK activation, which suggests that while PKC was shown to be the major secondary messenger of CCN2-mediated signals, the PKC–p38 pathway was independent of JNK signalling [78].

In contrast, based on the results of Kim and colleagues, a link could be established between PKC and JNK signalling in chondrocytes [10]. They used a different approach: microRNAs (miRNAs). These small regulatory RNAs have been implicated in various cellular processes such as cell fate determination, cell death, and differentiation. In this study, they investigated the role of miRNA-34a in the reorganisation of the actin cytoskeleton, which is essential for chondrocyte differentiation as previously discussed, and found that in chicken chondroprogenitor cells treated with JNK inhibitor—which suppresses chondrogenic differentiation—the expression level of miRNA-34a was upregulated. On the other hand, blockade of miRNA-34a was associated with decreased protein expression of RhoA, a known modulator of stress fibre expression and consequent downregulation of stress fibres, and recovery of type II collagen expression [10]. Since miRNA-34a negatively modulates reorganisation of the actin cytoskeleton, which is one of the essential processes for establishing the chondrocyte phenotype; and since PKC is a key mediator of actin cytoskeleton reorganisation, these results suggest
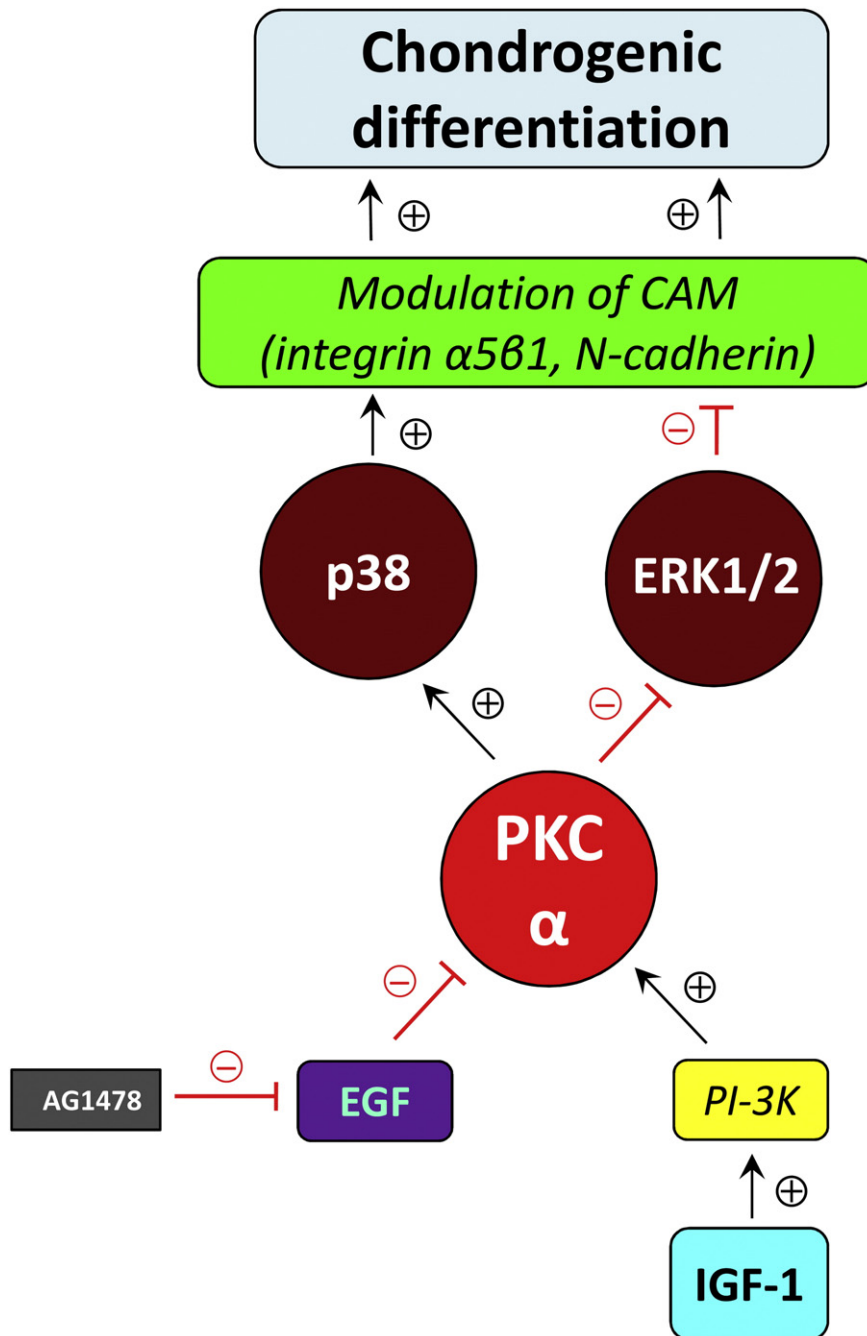
Fig. 9. Illustration of IGF-1 and EGF pathways which converge on and differentially regulate PKCα, a central signalling relay in chondrogenic cells. IGF-1 upregulates PI-3K and thus activates PKCα, which promotes chondrogenesis as described earlier. EGF, on the other hand, inhibits chondrogenesis by blocking PKCα. The chondro-inhibitory effect of EGF could be prevented by the EGFR blocker AG1478. Further details in text.

a putative link between JNK, miRNA-34a and PKC—although further studies are needed to confirm this hypothesis.

7. Involvement of PKC in other pathways during chondrogenesis

Since many open questions remained, endeavours to decipher the precise role of PKC isoforms in regulating cartilage formation have continued. As mentioned above, the PKA pathway has long been established to be a positive regulator of *in vitro* chondrogenesis [79]. As an attempt to find cross-talk between PKA and PKC pathways, Yoon and colleagues undertook to carry out further experiments. In that study, PKA was found to regulate N-cadherin expression in differentiating chondrocytes

by regulating PKCα: inhibition of PKA with H89 blocked the activation of PKCα; moreover, inhibition or downregulation of PKC isoforms was all accompanied by the same altered pattern of N-cadherin expression [21]. Furthermore, PKA was found to be an upstream regulator of PKCα as selective inhibition of PKCs did not affect PKA activity (see Fig. 10).

As mentioned previously, PKC was involved in mediating the effects of glucose concentration in culture medium [68]. Since the glucose effect has also been shown to have a profound effect on mesenchymal stem cell (MSC) differentiation, Tsai and co-workers aimed to evaluate the extent of chondrogenesis of MSCs previously cultured with different concentrations of glucose [80]. In that study, MSCs maintained in high glucose were less chondrogenic than low glucose maintained cells.
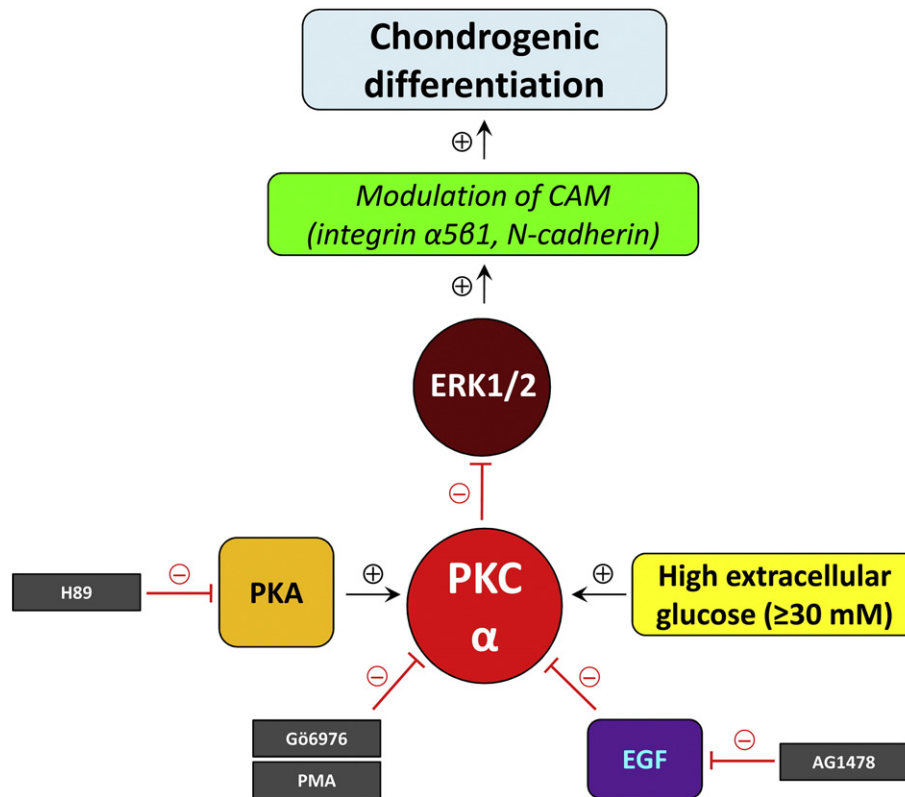
Fig. 10. PKC isoenzyme-dependent (primarily PKCα) regulation of cell adhesion molecules (integrin α5β1, N-cadherin) that are essential for chondrogenesis. PKCα activity is required for deactivation of ERK1/2, a negative regulator of chondrogenic differentiation in chicken limb bud-derived micromass cultures, which is in turn essential for the modulation of cell adhesion molecule expression. PKA is an upstream activator for PKCα since its inhibition with H89 altered the signalling pathway. High concentrations of extracellular glucose also activate this pathway and thus enhance chondrogenesis. By contrast, EGF negatively regulates this signalling since the EGFR blocker AG1478 prevented the negative effects of EGF. For a more detailed explanation, please see text.

Moreover, culture in high glucose-containing medium increased the phosphorylation of PKC and expression of type II TGF-β receptor. Inhibiting the PKC activity of high glucose-maintained MSCs with Gö6983 upregulated type II TGF-β receptor expression of chondrogenic cell pellets and enhanced chondrogenesis. In summary, these data suggest that the chondrogenic capacity of MSCs is modulated by the glucose concentration of the medium in expansion culture *via* PKC-dependent signalling, and that chondrogenesis induced in subsequent pellet culture can be enhanced by modulating PKC activity (see Fig. 6) [80].

8. PKC-dependent regulation of chondrogenesis *via* cell adhesion molecules

Early steps of chondrogenesis are widely accepted to be controlled by cell-to-cell and cell-to-ECM interactions through coordinating precartilage condensation and cartilage nodule formation. Several cell adhesion molecules such as N-cadherin and integrins (integrin α5β1 in particular) are reported to be involved in this process [74]. N-cadherin expression is downregulated during progression from precartilage condensation to cartilage nodule formation during chondrogenic differentiation, and it is inversely correlated to that of type II collagen, suggesting a determining role for this cell adhesion molecule. Based on the central role of PKC signalling that is indispensable for chondrogenesis to occur, several attempts have been made to establish a link between PKC and cell adhesion molecules.

As previously mentioned, Chang and co-workers proposed that PKC regulates chondrogenesis by modulating N-cadherin expression *via* ERK-1 activity [67]. They demonstrated that inhibition of PKC activity by Gö6976 or chronic exposure to PMA modulated the expression of cell

adhesion molecules (N-cadherin, integrin) involved in precartilaginous condensation during early chondrogenesis, which was reversed by the inhibition of ERK-1 by PD98059. From these data, the authors concluded that ERK-1 regulates the expression of cell adhesion molecules and ECM components in a PKC-dependent manner. To shed more light on this pathway, Yoon and co-workers demonstrated that PKA, a quintessential regulator of chondrogenesis, regulates N-cadherin expression in differentiating chondrocytes through PKCα as inhibition of PKA with H89 blocked the activation of PKCα [21]. Furthermore, as discussed above, stimulation of the EGF receptor blocked precartilage condensation and altered the expression of cell adhesion molecules (N-cadherin and integrin α5β1) by inhibiting the expression and activation of PKCα with concurrent activation of ERK-1 and inhibition of p38; all these effects could be blocked by the application of the EGF receptor inhibitor AG1478 [77].

The PKC-dependent regulation of key cell adhesion molecules was also confirmed by another study. As previously mentioned, Han and co-workers observed that high concentrations of glucose significantly stimulated chondrogenesis [68]. They reported that glucose modulated the expression patterns of cell adhesion molecules including fibronectin, integrin β1, and N-cadherin and concluded that by acting through PKCα high doses of glucose induce the downregulation of ERK1/2 activity and stimulate chondrogenesis *via* modulating the expression of adhesion molecules [68].

The above results indicate that PKC and ERK1/2 regulate the expression of cell adhesion molecules during chondrogenesis of mesenchymal cells. However, it turned out that during chondrocyte de- and redifferentiation, their relationship is different. Yoon and co-workers observed that the low levels of N-cadherin in differentiated rabbit articular chondrocytes dramatically increased as the differentiated phenotype was lost by serial monolayer culture; upon redifferentiation

of dedifferentiated cells, the elevated N-cadherin expression returned to the previous levels. However, inhibition of PKC with long-term exposure to PMA in chondrocytes cultured in monolayer did not affect N-cadherin expression, nor did it alter N-cadherin levels in dedifferentiated cells cultured in alginate gel [74]. These data collectively indicate that unlike during the differentiation process, PKC (and ERK1/2) does not regulate de- and redifferentiation of articular chondrocytes by altering the expression of cell adhesion molecules.

The results of these studies on the role of PKC signalling in mediating the expression of cell adhesion molecules during chondrogenesis are shown in Fig. 10.

9. PKC signalling during pathological conditions

Shortly after the expression and activity of PKC isoenzymes have been reported in model systems to study *in vitro* chondrogenesis, attempts have been made to generate data on the role of PKCs in disease progression. Osteoarthritis (OA), one of the ten most disabling diseases in developed countries, is the most common form of musculoskeletal disorders, mainly caused by age, genetic predisposition, obesity and prior joint injuries. OA is characterised by inflammation and breakdown of the shock-absorbing articular cartilage within the joint, and consequently symptoms including pain, swelling, inflammation, bone spur formation, and impaired mobility result [81]. To date, no effective or curative treatment is available for OA other than management of pain with analgesics and inflammation with non-steroidal anti-inflammatory drugs (NSAIDs). Current therapies are primarily targeted at reducing pain and inflammation. Therefore, there is a pressing socio-economic need for the development of therapeutic strategies to preserve the natural articular cartilage and its underlying bone. To achieve this, a better understanding of the cellular background of the disease is inevitable. It is therefore not surprising that considerable efforts have been made to identify alterations in key signalling pathways such as PKC signalling in inflammatory chondrocytes. Furthermore, the PKC signalling pathway is a major drug target because several isoforms are important regulators of survival or apoptosis, attempts have been made to establish a link between PKC signalling and degenerative joint diseases.

9.1. PKC isoforms with altered expression patterns in healthy and OA chondrocytes

One of the earliest publications in this field by Satsuma and colleagues in 1996 described altered expression patterns of various PKC isoenzymes in healthy and mechanically induced OA chondrocytes in rats [82]. They found that cells expressing PKCβ and PKCγ could only be identified in healthy chondrocytes but not in sham-operated or OA samples. Quite interestingly PKCα, which was only observed in the subchondral bony layer in the controls, appeared in chondrocytes in the superficial and columnar layers of the osteoarthritic knee. Although PKCε expression was detectable in chondrocytes of the superficial portion of the columnar layer in the control, signals for PKCε disappeared from the superficial region of columnar layer and increased in the middle of the columnar layer in early OA joints. However, as this was just a descriptive study, the authors were unable to establish whether the observed redistributions of PKC isoenzymes were signs for initiating destructive processes, reflected attempted cell repair mechanisms, or were simply a consequence of alterations in cellular signalling pathways.

The same group continued their research interests and by using an experimental model of slowly progressive osteoarthritis in rabbits, they reported that intra-articular administration of the PKC-activating phorbol ester TPA almost completely blocked the progression of OA, whereas a non-PKC-activating phorbol ester (phorbol-12,13-didecanoate, PDD) failed to prevent OA [83]. In a different study, they once again observed that PKCα could be detected in chondrocytes from experimentally induced OA cartilage and that intra-articular administration of TPA

prevented the development of the disease; furthermore, they also provided evidence that PKC was involved in the stress-mediated degradation of articular cartilage [84].

In contrast to these initial results, LaVallie and co-workers demonstrated the presence of different PKC subtypes in arthritic chondrocytes [51]. As discussed above, they were the first to apply Affymetrix GeneChip® to study differences in the transcriptome of healthy and osteoarthritic cartilage samples. Of the four PKC isoenzymes detected in human chondrocytes, only PKCζ appeared to be transcriptionally altered in OA articular cartilage compared with normal articular cartilage, with an approximately 2.5–3.5-fold upregulation of transcript levels in OA cartilage. Nevertheless, these observations served as a basis of the chondroprotective hypothesis for PKC activation during early stages of OA.

9.2. Molecular dissection of PKC signalling pathways in inflammatory chondrocytes

In support of this theory, further experiments were conducted. Since one of the major factors in OA onset and progression is extensive mechanical load on major weight bearing joints, Fukuda and coworkers examined the effects of mechanical stress on chondrocyte metabolism and the activity of PKC [85]. They reported that low frequency and magnitude of cyclic tensile stretch increased proteoglycan synthesis in chondrocytes, whereas high frequency and magnitude loading decreased this parameter. Moreover, in this condition, PKC activity was reduced. The authors suggested the involvement of PKC in the mechanical stress-mediated inhibition of proteoglycan synthesis, probably *via* modulating the actin cytoskeleton.

Mediated primarily by cytokines such as IL-1, IL-17, and tumour necrosis factor α (TNFα), inflammation is a major characteristic of joint diseases such as OA, during which inflammatory mediators are released by infiltrating immune cells. It is generally accepted that in chondrocytes, the effects of these pro-inflammatory cytokines such as IL-1 are mediated through NO production. NO regulates cartilage degradation by causing dedifferentiation and apoptosis of chondrocytes *via* activation of ERK1/2 and p38, respectively [86]. Since PKC plays a central role in transducing signals that influence differentiation, survival and apoptosis, Kim and colleagues investigated the molecular mechanisms of how PKC isoforms regulate NO-induced dedifferentiation and apoptosis of articular chondrocytes. Indeed, they found that activities of PKCα and PKCζ were reduced during NO-induced dedifferentiation and apoptosis; while the attenuated PKCα activity was independent of ERK1/2 or p38, PKCζ activity was reduced as a result of NO-induced p38 kinase activation [61]. Furthermore, the fact that inhibition of PKCα and PKCζ activities potentiated NO-induced apoptosis, whereas ectopic expression of these isoforms significantly reduced the number of apoptotic cells and blocked dedifferentiation further supports the theory that PKC isoforms are involved in mediating NO-induced signalling. Thus, these results indicate that p38 kinase-independent and -dependent inhibition of PKCα and PKCζ, respectively, regulates NO-induced apoptosis and dedifferentiation of articular chondrocytes [61]. Moreover, as p38 kinase also regulates NF-κB, Kim and Chun investigated the functional relationship between PKC and NF-κB signalling in chondrocytes and reported that although NO-stimulated NF-κB activation was inhibited by ectopic PKCα and PKCζ expression, NO-induced inhibition of PKCα and PKCζ activity was not affected by NF-κB inhibition, indicating that NO-induced inhibition of PKCα and PKCζ activity was required for the NF-κB activity that regulates apoptosis but not dedifferentiation in articular chondrocytes [87].

Kim and colleagues further dissected the molecular mechanism regulating chondrocyte dedifferentiation and apoptosis by investigating the effects of the actin cytoskeletal architecture on NO-induced dedifferentiation and apoptosis in articular chondrocytes, with a focus on ERK1/2, p38 and PKC signalling [62]. Disruption of the actin cytoskeleton by cytochalasin D inhibited NO-induced apoptosis and dedifferentiation

in chondrocytes without affecting ERK1/2 activity but blocking activation of p38 kinase and inhibition of PKCα and PKCζ. Furthermore, cytochalasin D also suppressed downstream signalling of p38 kinase and PKC, such as NF-κB activation and p53 accumulation, which are necessary for NO-induced apoptosis. There results indicated that in accordance with the above data, the actin cytoskeleton mediates NO-induced regulatory effects in chondrocytes by modulating p38, as well as PKCα and PKCζ [62] (see also Fig. 5).

IGF-1 is known to inhibit the catabolic effects of IL-1 on PG synthesis in cartilage explants and suppresses the degradation of ECM components by reducing matrix metalloproteinase-1 (MMP-1) and MMP-8 expression and activity. In addition, it has long been known that levels of IGF-1 are enhanced in the synovial fluid of OA patients [88], and that the response of inflammatory chondrocytes to IGF-1 is reduced [89], suggesting that IGF-1 is involved in the pathophysiology of OA. Based on that, Oh and Chun examined the role of IGF-1 in NO-induced dedifferentiation and apoptosis of articular chondrocytes and reported that IGF-1 inhibits NO-induced dedifferentiation and apoptosis [76]. As mentioned earlier, IGF-1 induces chondrogenic differentiation *via* the PI-3K–PKCα–p38 pathway (see also Fig. 9). In addition, they have also shown that IGF-1 inhibits NO-induced dedifferentiation and apoptosis of articular chondrocytes through the activation of PI-3K and by conversely regulating p38, ERK1/2, as well as PKCα and PKCζ [76]. In summary, the effects of IL-1 mediated through NO production regulate differentiation, survival, and inflammatory responses by modulating ERK1/2, p38, as well as PKCα and PKCζ in articular chondrocytes during OA.

In terms of MMP-2 and MMP-9 secretion, Chu and co-workers applied multiple inhibitors in early arthritic chondrocytes including PKC inhibitors such as staurosporine, compound H7, and Gö6976 [90]. They demonstrated that the PKC inhibitors suppressed both MMP-2 and MMP-9 production in OA chondrocyte cultures, which implicate that PKC signalling was involved in disease onset and progression in early OA.

LaVallie and colleagues demonstrated that PKCζ was transcriptionally upregulated in human OA articular cartilage clinical samples [51]. Furthermore, upon IL-1 treatment rapidly increased phosphorylation of PKCζ was observed, implicating its activation and function in the signalling pathway. In primary chondrocytes, application of IL-1 and TNFα increased NF-κB activity resulting in proteoglycan degradation, which could be blocked by the PKC inhibitors RO31-8220, bisindolylmaleimide I and Gö6976, but at the same time it was unaffected by calphostin C that does not inhibit atypical PKCs including PKCζ. Moreover, either a cell-permeable PKCζ pseudosubstrate peptide inhibitor or overexpression of a dominant negative PKCζ protein could prevent TNFα and IL-1-mediated NF-κB activation and proteoglycan degradation. These findings support the hypothesis that PKCζ may be an important signalling component of cytokine-mediated cartilage ECM degradation during OA [51], in agreement with the results discussed above [62].

A year later, the same team further analysed the role of PKCζ in OA onset and progression and reported that the phosphorylation of PKCζ and NF-κB induced by treatment with IL-1 could be specifically blocked by inhibitors of atypical PKCs [91]. Moreover, inhibition of PKCζ suppressed IL-1-mediated upregulation of ADAMTS-4 and aggrecanase, whereas inhibitors of atypical PKCs also blocked IL-1-induced NO production and NOS2 mRNA expression, demonstrating a link between PKCζ and NO production in OA chondrocytes. Furthermore, transient knock-down of PKCζ mRNA significantly reduced both ADAMTS-4 and NOS2 mRNA expressions, indicating that PKCζ is involved in the regulation of IL-1-induced NF-κB signalling in human OA chondrocytes [91]. Therefore, these results suggest that PKCζ regulates the production of matrix-degrading enzymes as well as NO production in OA and may have a profound effect on disease progression.

As mentioned earlier, apart from PKCζ, PKCα has also been implicated as a major player in OA pathogenesis. To this end, Chen and colleagues investigated the expression of PKCα in chondrocytes of

medium or late stage human knee OA cartilage to further evaluate their role in chondrocyte apoptosis during disease progression [92]. They provided further data in support of the early observations of Satsuma and colleagues [82] in that the increased expression of PKCα was involved in OA progression and apoptosis, and concluded that the correlation between OA progression, the role of PKCα-dependent signalling pathways, and chondrocyte apoptosis allows for new therapeutic strategies to be considered. The concept of PKC isotypes mediating OA onset and disease progression along with a suggested signalling pathway and the scientific data in support of this hypothesis is shown in Fig. 11.

10. Conclusions

In summary, it is evident that we are only at the beginning of understanding the precise role of PKC subtypes during chondrogenesis and how they are regulated. Clearly, there are many yet unexplored fields in this area; by exploiting these novel approaches and applying them to not only healthy but also inflammatory chondrocytes may enable us to halt or even reverse disease progression. Shedding more light on exactly how PKC signalling pathways and Ca^{2+} homeostasis interact with each other; or elucidating novel miRNA-mediated control mechanisms would open new perspectives for a better understanding of healthy as well as pathological differentiation processes of chondrocytes, and may also lead to the development of new therapeutic approaches and novel strategies for controlling the differentiation of MSCs.

11. Future directions

The formation of cartilage that makes up the majority of the vertebrate skeleton at the beginning of embryonic development relies on a delicate sequence of interactions between differentiating chondroprogenitor cells and a plethora of external stimuli such as various growth factors, morphogens, hormones, cytokines, and many other ligands. We are only beginning to understand the key pathways through which these multiple stimuli are decoded and integrated to coordinate cellular responses and changes in gene expression events that are characteristic to the particular stage of chondrocyte maturation within the developing multicellular organism. From the pieces of evidence we have reviewed here, it is evident that PKC isoforms, especially PKCα, are central players in these processes (Fig. 12). However, our current understanding of the precise mechanisms through which PKC signalling regulates chondrogenesis and chondrocyte function is still extremely limited.

11.1. PKC and Ca^{2+} signalling

Although the downstream components of PKC signalling during chondrogenesis such as ERK1/2 and p38 MAPK, cell adhesion molecules, changes in gene expression of key chondrogenic markers are relatively well-known, current knowledge concerning signalling components located upstream of PKC are only poorly characterised. Given that PKCα is a prototypical member of the classic PKC isoenzymes, cytosolic Ca^{2+} should be considered as a major regulator. In general, cellular events governed by Ca^{2+} require transiently elevated cytosolic Ca^{2+} concentration for Ca^{2+}-sensitive signalling components to be activated [93]. There are two main sources of Ca^{2+} for initiating and generating signals; Ca^{2+} entry across the plasma membrane *via* either voltage-operated Ca^{2+} channels or agonist-dependent and voltage-independent Ca^{2+} entry pathways; and Ca^{2+} release from internal stores such as the smooth endoplasmic reticulum (ER) *via* the inositol-1,4,5-trisphosphate receptor (IP$_3$R) or the ryanodine receptor (RyR) [94]. Since changes of intracellular Ca^{2+} concentration are closely related to cell proliferation and differentiation that are important functions of undifferentiated cells such as chondroprogenitors or MSCs, the Ca^{2+} homeostasis of MSCs has been thoroughly investigated [95]. Furthermore, Ca^{2+} dependent signalling pathways are also key factors that govern chondrogenesis [96].
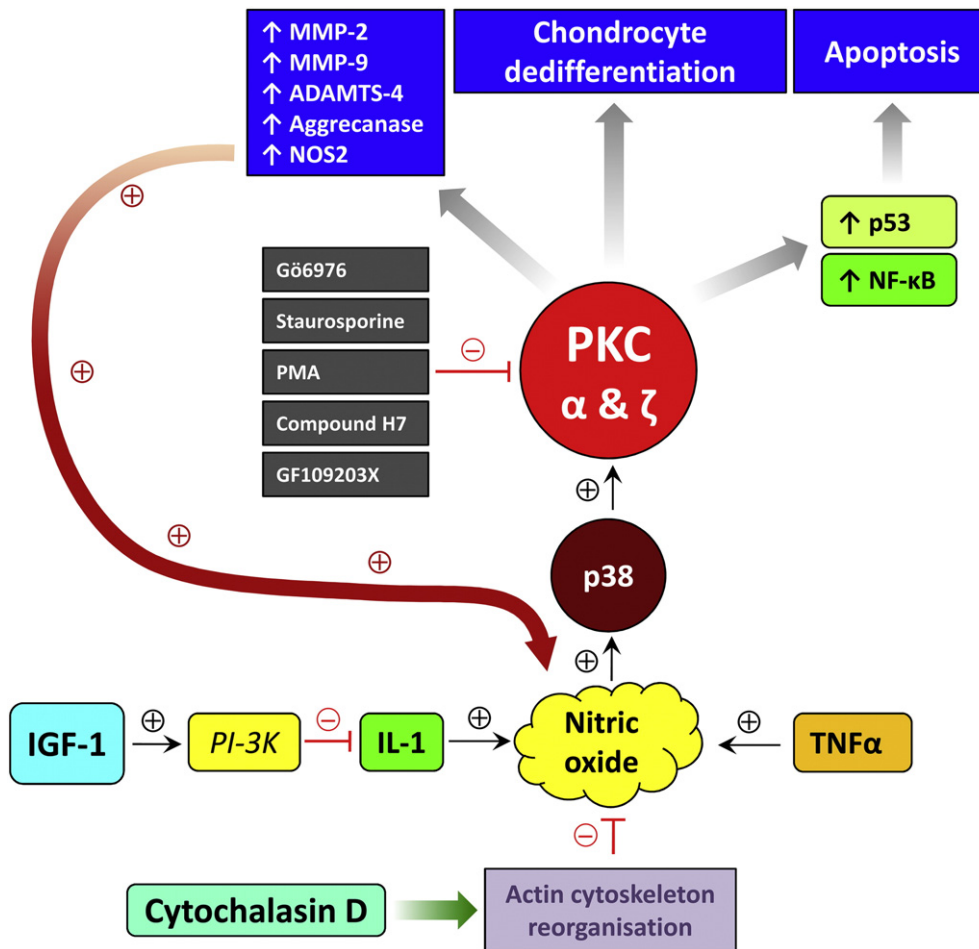
Fig. 11. PKCα and PKCζ are key components in signalling pathways that are suggested to be involved in OA pathogenesis and progression. NO is a key mediator liberated during inflammatory processes, whose production is enhanced by TNFα and IL-1, but blocked by cytochalasin D-induced cytoskeletal rearrangement. The effects of IL-1 could be blocked by the IGF-1 pathway. Nitric oxide exerts its detrimental effects such as chondrocyte apoptosis and dedifferentiation, as well as activation of cartilage ECM degrading enzymes (MMP-2, MMP-9, ADAMTS-4, aggrecanase) *via* signalling that involves p38 MAPK, PKCα and PKCζ, NF-κB and p53. Furthermore, this pathway activates NOS2 that is involved in NO generation. Please see text for a more detailed explanation and references.

It is well documented that different spatial and temporal patterns of intracellular free Ca^{2+} concentration play distinct roles in the regulation of various cellular processes. The same applies to *in vitro* chondrogenesis; we have reported that a distinct pattern of cytosolic Ca^{2+} concentration was required for the differentiation of chicken limb bud-derived mesenchymal cells in micromass cultures; this parameter was found to be very precisely regulated in chondroprogenitor cells since altering resting Ca^{2+} levels by either A23187 Ca^{2+} ionophore or EGTA over or below certain threshold values abrogated chondrogenesis [97]. Furthermore, we managed to establish a correlation between basal cytosolic Ca^{2+} concentration and activity of the Ca^{2+}–calmodulin dependent phosphoprotein phosphatase calcineurin, a key regulator of *in vitro* chondrogenesis in chicken limb bud-derived micromass cultures. Conversely, inhibition of calcineurin by cyclosporine-A altered resting Ca^{2+} levels in differentiating chondroprogenitors, implicating a reciprocal regulation between Ca^{2+} homeostasis and Ca^{2+}-sensitive effector proteins [97].

Although in that study, we have not looked at how PKC activity might be modulated by altered basal intracellular Ca^{2+} levels, nor did we investigate if there was an effect of selective inhibition of PKCα on the Ca^{2+} homeostasis, it would be interesting to establish such a link during chondrogenesis. Since according to Sonn and Solursh, PKC activity was initially low but significantly increased during the course of chondrogenesis in limb bud-derived HDC [42]; furthermore, we found that there is a rise in resting intracellular Ca^{2+} levels during

chondrogenesis, we can speculate that changes in PKC activity can at least partially be attributed to the characteristic pattern of basal Ca^{2+} concentration and conversely, how PKC activity may modulate basal Ca^{2+} levels. In fact, such a correlation has long been known to exist in Jurkat T cells in which a PKC-sensitive Ca^{2+} extrusion mechanism has been reported; furthermore, Balasubramanyam and Gardner described that activation of PKC modulated intracellular Ca^{2+} levels by both inhibiting Ca^{2+} influx and by stimulating Ca^{2+} extrusion [98]. In vascular smooth muscle cells, the potent vasoconstrictor substances 5-hydroxytryptamine, angiotensin II, and bradykinin that bind to G-protein coupled receptors and activate PLC-β induced a transient increase in intracellular calcium Ca^{2+} and at the same time brought about a rapid but transient activation of the calcium sensitive PKCα, which resulted in increased proliferation rate [99]. In concert with these findings, an increase in cytosolic Ca^{2+} levels and concurrently elevated PKC activity may act cooperatively on the myogenic contraction of skeletal muscle arteries [100]. Correlation between increased free cytosolic Ca^{2+} levels and enhanced PKC activity has also been found in rat cerebrocortical presynaptic nerve terminals [101]. Sohen and co-workers reported that in chondrocytes, the activation of the histamine H1 receptor led to stimulation of proteoglycan synthesis and evoked increases of intracellular Ca^{2+} levels and that PKC activity was also enhanced parallel to H1 receptor activation; moreover, PKC inhibitors antagonised histamine-stimulated proteoglycan synthesis, which suggested that PKC signalling was involved in H1 receptor-mediated stimulation of proteoglycan synthesis in mature articular
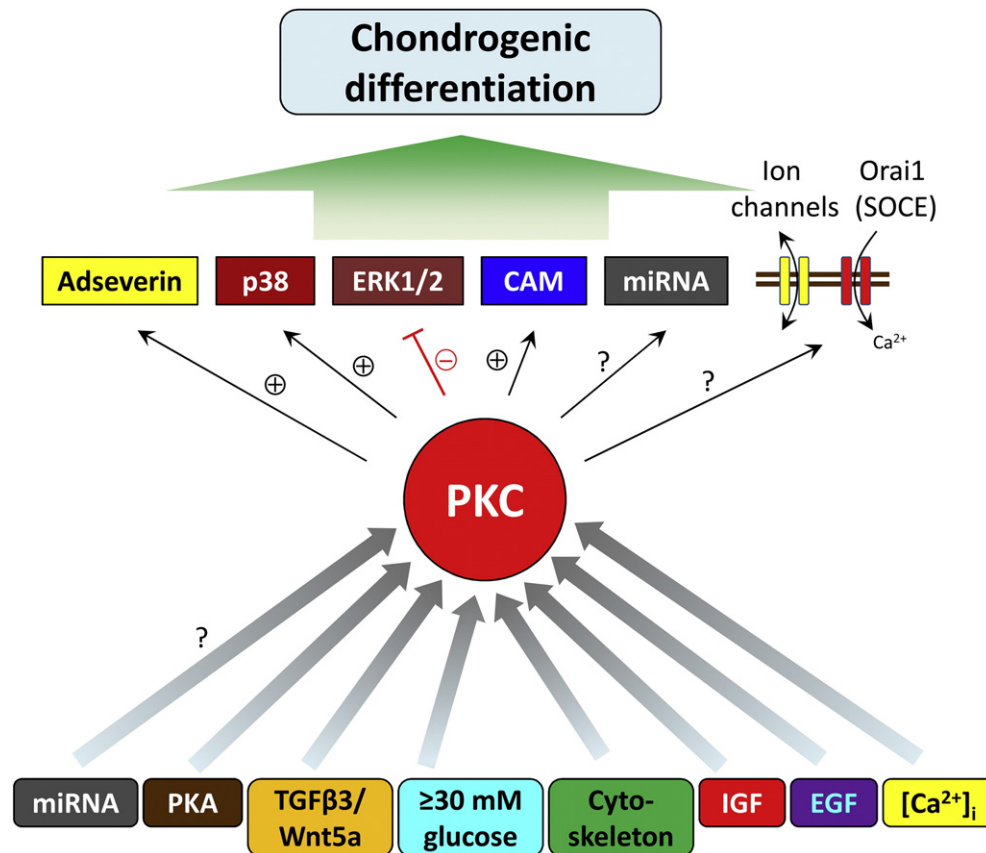
Fig. 12. Summary of upstream modulators and downstream effectors of PKC signalling during chondrogenesis. Putative factors upstream and/or downstream of PKC that have not been confirmed in differentiating chondroprogenitors are shown with a question mark. (CAM, cell adhesion molecules).

chondrocytes [102]. Therefore, it would be worth studying how these two pivotal regulators of chondrogenesis, *i.e.* PKC signalling pathways and Ca^{2+} homeostasis, are interconnected with each other during chondrogenic differentiation.

12. PKC as a molecular decoder of Ca^{2+} oscillations

Besides a stable rise, periodic oscillatory changes of cytosolic Ca^{2+} concentration also represent a nearly universal signalling mechanism even in non-excitable cells [103]. Depending on the frequency and amplitude of the Ca^{2+} transients, Ca^{2+} oscillations are known to control cellular differentiation *via* activating different Ca^{2+}-dependent transcription factors including nuclear factor of activated T lymphocytes (NFAT), NF-κB, JNK1, and CREB [104], most of which are key regulators of chondrogenic differentiation. In our previous studies, we analysed intracellular Ca^{2+} dynamics in individual chondroprogenitor cells at high spatial and temporal resolution and established that differentiating cells in chondrifying chicken micromass cultures were characterised by rapid spontaneous Ca^{2+} oscillations modulated by voltage gated potassium channel (K_V)-driven membrane potential changes as well as voltage gated Ca^{2+} channels [105,106]. Apart from voltage operated ion channels in the plasma membrane, contribution from the internal Ca^{2+} stores was also indispensable to these spontaneous Ca^{2+} events as blockade of store-operated Ca^{2+} entry (SOCE) abolished repetitive Ca^{2+} transients and abrogated *in vitro* chondrogenesis primarily *via* reducing proliferation rate, suggesting a key regulatory role of these processes [105].

That classic PKC isoforms can act as a molecular decoding machine for Ca^{2+} signals has long been known; it has been reported that the frequency of Ca^{2+} spikes can tightly control the extent and timing of PKC activation in rat basophilic leukaemia 2H3 cells [27]. Interestingly,

it has also been found that maximal and persistent PKC activation could only be reached when high-frequency Ca^{2+} oscillations were concurrently present. This enables us to speculate that such a scenario could exist in differentiating chondroprogenitors; given that altering the amplitude and frequency of spontaneous Ca^{2+} spikes leads to abrogation of *in vitro* chondrogenesis, one of the possible downstream effectors of Ca^{2+} oscillations could be the activation of classic PKC subtypes. Although this putative link between repetitive Ca^{2+} signals and PKC activation has not been tested during early steps of chondrogenesis, it would be intriguing to see how modulation of such Ca^{2+} events affect PKC activation and conversely, how various parameters of Ca^{2+} spikes are modulated following administration of PKC inhibitors. There is evidence that PKC is involved in the regulation of ATP-triggered Ca^{2+} oscillations in chicken granulosa cells; PKC appeared to be a central player in these Ca^{2+} events as oscillations were prevented by either full activation or inhibition of PKC activity [107].

13. Ion channels as downstream targets of PKC

Although chondrocytes are non-excitable cells, their plasma membrane contains a rich complement of ion channels including a range of potassium channels (K_{ATP}, BK, K_V, and SK), sodium channels (epithelial sodium channels, voltage activated sodium channels), transient receptor potential calcium channels, various non-selective cation channels, chloride channels, which are collectively referred to as the chondrocyte channelome [108]. However, the function of these ion channels is less well understood; chondrogenesis, mechanotransduction, cell volume regulation, and apoptosis all appear to involve the function of ion channels and thus their function is indispensable to chondrocyte function [109]. Therefore, modulation of chondrocyte ion channel activity is of central importance for cartilage formation and maintenance. PKC has

been well known to modulate ion channels: it inhibits Ca^{2+}-activated K^+ channels and K_{ATP} channels [110,111], and modulates voltage gated K^+ [112], Ca^{2+} [113], and Cl^{2-} channels [114]. Apart from *bona fide* ion channels, PKC activity has been reported to be required for SOCE in human glomerular mesangial cells, since the thapsigargin-induced Ca^{2+} entry pathway was abolished by the PKC inhibitor calphostin C, whereas the PKC activator PMA enhanced Ca^{2+} influx [115]. However, since the molecular identity of the SOC channel has not been revealed at that time, they were unable to unequivocally identify the protein whose phosphorylation was required for SOCE.

Although the mechanism of SOCE has long been known, the molecular identity of the channel-forming proteins was revealed only recently [116]. SOCE is initiated by oligomerisation of the Ca^{2+} sensor stromal interaction molecules STIM1 and STIM2 in the endoplasmic reticulum (ER) membrane, triggered by a decrease in the Ca^{2+} content below a certain threshold level. Once oligomerised, STIM1/STIM2 is redistributed into distinct puncta in the ER membrane that are located in close proximity to the plasma membrane, where STIM interacts with Orai1 dimers enabling them to form tetramers, thus giving rise to the functional Ca^{2+} channel. SOCE has been shown to be indispensable for chondrogenesis to take place as administration of SOCE inhibitors abrogated cartilage matrix formation in micromass cultures; furthermore, SOCE was also required for repetitive Ca^{2+} oscillations, a phenomenon that was found to be necessary for the differentiation of chondroprogenitor cells [105]. However, the regulation of SOC channels is largely unknown. It was not until 2010 that Kawasaki and colleagues suggested a mechanism by which PKC regulates SOCE in HEK293 cells. In that study, they demonstrated that PKC suppressed SOCE by phosphorylating Orai1 at N-terminal Ser-27 and Ser-30 residues, since application of PKC inhibitors and knockdown of PKCβ both resulted in increased Ca^{2+} influx [117]. The fact that Ma and co-workers found that PKC activation was required for SOCE in mesangial cells contradicts with the findings of Kawasaki and colleagues who found that phosphorylation of Orai1 by PKC suppressed Ca^{2+} entry might be resolved by assuming that regulation of SOCE by PKC is likely to be dependent on the PKC isoforms and types of SOC channels expressed in any given cell type. Therefore, it would be intriguing to investigate whether PKC is involved in the regulation of SOCE in differentiating chondroprogenitor cells and thus modulate Ca^{2+} oscillations to promote chondrogenesis.

14. miRNA-mediated PKC regulation of chondrogenesis

Apart from Ca^{2+} signalling, there are other pathways potentially involved in mediating PKC activity during chondrogenesis. One such possibility might be regulation through miRNAs, which are evolutionarily conserved small non-coding RNAs that modulate gene expression and play important roles in diverse biological functions, including cell differentiation (for a relevant recent review, see [118]). Recent studies have indicated that miRNAs play important roles in tissue morphogenesis, and increasing evidence indicates that miRNAs are an integral part of the regulatory network in chondrocyte differentiation and cartilage function. In particular, miRNA-145 was reported to downregulate *Col2a1*, *Acan1*, *COMP*, *Col9a2*, and *Col11a1*, and reduces GAG content during chondrogenic differentiation of MSCs, which indicate that miRNA-145 is a key negative regulator of chondrogenesis, directly targeting Sox9 at an early stage of the process [119]. The laboratory of Jin has been carrying out research that also identified miRNAs as key regulators of various steps of chondrogenesis ranging from maintenance of cartilage integrity [120], migration of chondroprogenitor cells [121], cytoskeletal dynamics [10], and also during osteoarthritis [122]; in fact, based on the results of this group and others, miRNAs are emerging new targets for OA.

As mentioned above, miRNA-34a has been reported to negatively modulate reorganisation of the actin cytoskeleton, which is essential for establishing chondrocyte-specific morphology [10]. We herein discussed earlier that PKC signalling was central to mediating the effects

of cytoskeletal reorganisation during chondrogenesis, as well as chondrocyte re- and dedifferentiation. Assuming that both miRNA and PKC signalling converge on the actin cytoskeleton, it would be logical to hypothesise an miRNA-mediated control of PKC. Although such a regulatory pathway has not been reported in chondrocytes, miRNA-mediated regulation of PKC activity [123] and conversely, PKC-controlled miRNA activity [124] has been demonstrated in other systems. Given that other kinases, PKA in particular, have been found to be regulated by miRNA-23b during chondrogenesis in MSCs [125], it is plausible to assume that such regulation for PKC subtypes also exists.

14.1. Implications of PKC and its modulation for tissue engineering applications

Due to their multipotent nature, hMSCs are increasingly being considered for clinical therapeutic applications, such as bone and cartilage tissue engineering. However, current differentiation protocols require further optimisation before they can be translated into the clinical practice. To achieve this, a deeper understanding of signalling pathways that control lineage-specific differentiation is inevitable to enable targeted manipulation and exploitation, which may enhance the quality of tissue engineered constructs. Given that PKC is a key regulator in chondroprogenitors, it can be a potential candidate to be targeted during hMSC differentiation. In fact, inhibition of conventional PKCs and activation of the novel PKCδ during osteogenic differentiation of hMSCs enhanced osteogenic differentiation both *in vitro* and *in vivo* [126]. Santos and colleagues reported that in adipose-derived stem cells (ADSCs) Wnt5a induced osteogenic differentiation *via* activation of the non-canonical Wnt signalling pathway by increasing PKC activity [127]. In a good correlation with these data, Park and Patel recently reported that overexpression of the PKCδ isoform in ADSCs resulted in a marked upregulation of cardiomyogenic genes such as Mef2C, cardiac actin and troponin, which indicates that PKCδ activation is a key regulator of cardiomyocyte formation [128]. PKC activation was also required to stimulate neurogenic differentiation of human stem cells of dental origin [129]. These important data suggest that targeted manipulation of PKC signalling pathways should be exploited to enhance the differentiation fate of MSCs, albeit further studies are needed to confirm the applicability of this approach in cartilage tissue engineering.

Conflict of interest statement

The authors disclose that there are neither any financial nor personal relationships with other people or organisations that could inappropriately influence (bias) their work. There are no conflicts of interests.

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