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Purinergic signalling-evoked intracellular Ca^{2+} concentration changes in the regulation of chondrogenesis and skeletal muscle formation

Csaba Matta[1,2], János Fodor[3], László Csernoch[3], Róza Zákány[1,*]

[1]*Department of Anatomy, Histology and Embryology, Faculty of Medicine, University of Debrecen, Debrecen, H-4032, Hungary*

[2]*Department of Veterinary Pre-Clinical Sciences, School of Veterinary Medicine and Science, Faculty of Health and Medical Sciences, University of Surrey, Guildford, GU2 7AL, United Kingdom*

[3]*Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, H-4032, Hungary*

*Corresponding author: Róza Zákány

E-mail: roza@anat.med.unideb.hu

Phone: +36-52-255-567

Fax: +36-52-255-115

Abstract

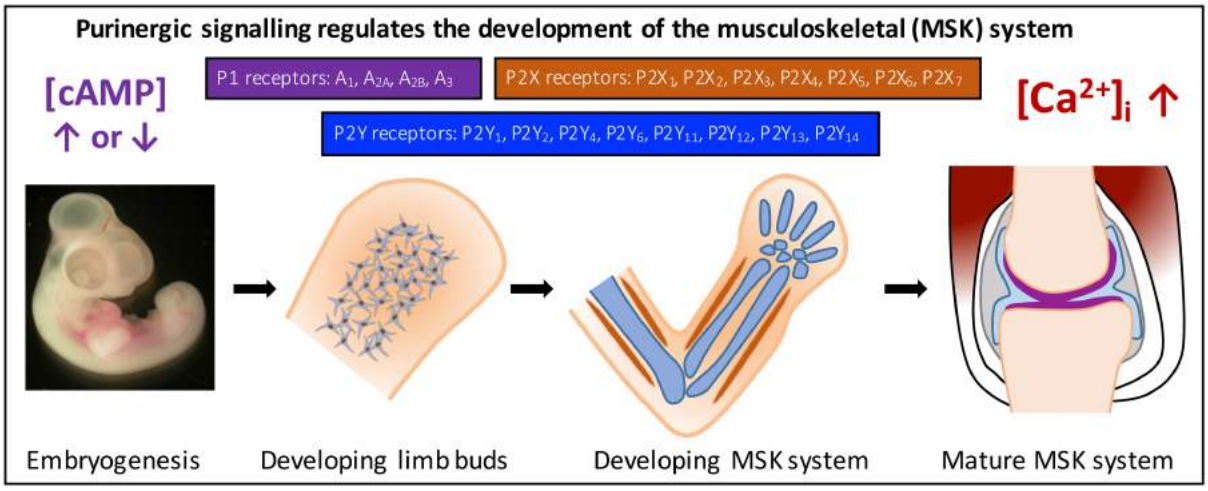
It is now widely recognised that changes of the intracellular calcium concentration have deep impact on the differentiation of various non-excitable cells including the elements of the vertebrate skeleton. It has become evident that purinergic signalling is one of the most ancient cellular mechanisms that can cause such alterations in the intracellular Ca^{2+}-homeostasis, which are precisely set either spatially or temporally. Purinergic signalling is believed to regulate intracellular Ca^{2+}-concentration of developing cartilage and skeletal muscle cells and suggested to play roles in the modulation of various cellular functions. This idea is supported by the fact that pluripotent mesenchymal cells, chondroprogenitors or muscle precursors, as well as mature chondrocytes all are capable of releasing ectonucleotides, and express various types of purinoreceptors and ectonucleotidases. The presence of the basic components of purinergic signalling proves that cells of the chondrogenic lineage can utilise this mechanism for modulating their intracellular Ca^{2+} concentration independently from the surrounding skeletal muscle and bone tissues, which are well known to release ectopurines during development and mechanical stress. In this review, we summarize accumulating experimental evidence supporting the importance of purinergic signalling in the regulation of chondrogenesis and during skeletal muscle formation.

(193 words in abstract)

Keywords

Cartilage formation; myogenesis; mesenchymal stem cells, purinoreceptor; ATP; Ca-oscillations

Graphical abstract



Abbreviations

AC, adenylate cyclase; ATSC, adipose tissue-derived stem cell; CPC, chondrogenic progenitor cell; DFC, dental follicle cell; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; FGF, fibroblast growth factor; GPCR, G-protein coupled receptor; HDC, high density cultures; IP_3, inositol 1,4,5-trisphosphate; MRF, myogenic regulatory factor; MSC, mesenchymal stem cell; MSK, musculoskeletal; NFAT, nuclear factor of activated T lymphocytes; NMDA, N-methyl-D-aspartate type ionotropic glutamate receptor; PI3-K, phosphoinositide 3-kinase; PLC, phospholipase C; PP2B, protein phosphatase 2B (calcineurin); RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic Ca^{2+}-ATPase; Shh, sonic hedgehog; SOCE, store operated calcium entry; TGF, transforming growth factor; TRP, transient receptor potential channel

1. Brief overview of purinergic signalling

Although the physiological role of extracellular purine nucleotides has been implicated early in the 20th century by Szent-Györgyi and his co-worker [1], it was not until the seminal work of Burnstock and colleagues on non-adrenergic non-cholinergic transmission in the *taenia coli* [2] that they gained proper acceptance into the growing community of signalling molecules. Since then purinergic signalling (coined by Burnstock [3]; see also [4]) has been widely accepted in nearly all eukaryotic organisms [5].

1.1. P1 receptors

The evolution of our understanding of purinergic signalling has first led to the separation of the then so-called P1 and P2 receptors based on their sensitivity to adenosine (P1 receptors) and ATP (P2 receptors) [6]. The former are now referred to as adenosine receptors, with four members of the family (A_1, A_{2A}, A_{2B}, and A_3) [7, 8] identified and characterised. All adenosine receptors belong to the large superfamily of G-protein coupled receptors (GPCRs) and affect the intracellular production of cyclic AMP (cAMP) or inositol 1,4,5-trisphosphate (IP_3). Whilst A_1 and A_3 receptors are linked in most cells to G_i proteins and thus lower cAMP concentration, both A_2 receptors couple to G_s proteins to increase intracellular cAMP levels [9, 10].

1.2. P2 receptors

Identification of the members of the P2 receptor family started with the description of their distinct and sometimes contradictory cellular effects [11]. The original notion in 1985 that P2X and P2Y receptors should be distinguished was based on pharmacological data [12]. Today seven members of the ionotropic ($P2X_{1–7}$) and eight members of the metabotropic ($P2Y_{1,2,4,6,11–14}$) purinergic receptors have been cloned and identified in mammals [13, 14]. In

case of P2X receptors the seven members of the subfamily refer to seven distinct subunits as discussed below. It should be noted that the missing numbers in the P2Y receptor family are for members either present in non-mammalian species (such as $P2Y_3$, which was found in birds) [15] or have since been recognized as receptors for other signalling molecules (such as the lysophosphatidylserine receptors $P2Y_5$ or $P2Y_{10}$) [16, 17]. The key components of P1 and P2 receptor-mediated signalling are illustrated in Fig. 1.

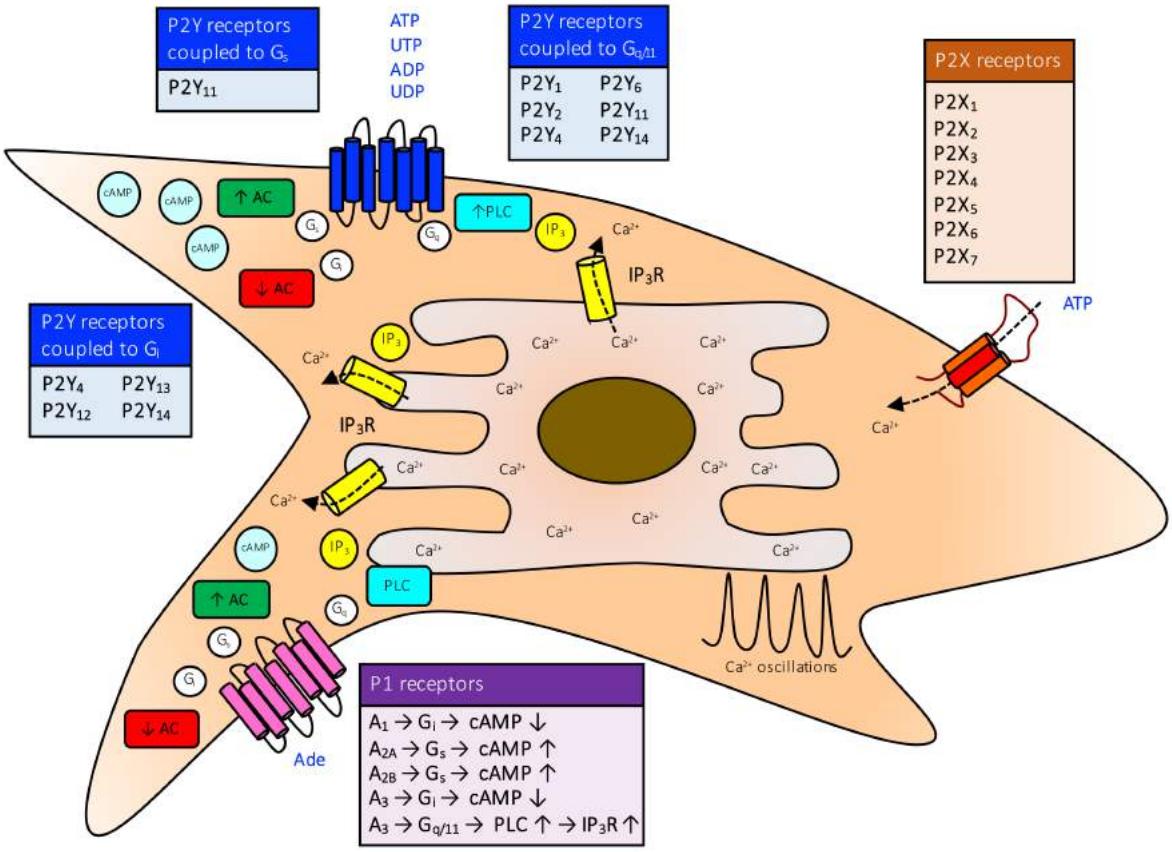


Figure 1. Schematic diagram depicting the molecular components of P1 and P2 receptor-mediated signalling in an idealised cell. The downstream effects of P1 adenosine and P2Y purinergic receptor subtypes is determined by the G protein; $G_{q/11}$ activates the PLC – IP_3R pathway, G_s activates AC and thus elevates cAMP levels, and G_i inhibits AC activity thus resulting in decreased cAMP levels. Please note that in reality, no cells express all types of P1 and P2 receptors that couple to all intracellular signalling pathways shown at the same time. AC, adenylate cyclase; PLC, phospholipase C.

1.2.1. P2X receptors

P2X receptors form multimeric, both homo- and heteromeric ligand gated channels composed of different members of the subfamily. Typical homomeric channels are the $P2X_1$ and $P2X_7$ receptors, while a typical heteromeric receptor is the one composed of $P2X_2$ and $P2X_3$ subunits (the $P2X_{2/3}$ receptor [18]). Recent data suggest that functional P2X receptors can occur as trimers [19, 20]. Nonetheless, all seven P2X receptor subunits share a similar topology, with two transmembrane domains, an extracellular loop, and the amino and carboxy terminals located intracellularly.

The ion channels formed by P2X receptors are cation selective. Their activation, therefore, results in the depolarization of the cell membrane. In certain cases the depolarization itself, in others the concomitant influx of calcium ions—and the consequent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)—is the cellular signal. Ca^{2+} ions enter either through the activated P2X receptor or the depolarization of the cell membrane results in the opening of voltage-gated calcium channels. In this respect P2X receptors can either be non-selective channels (such as the $P2X_3$ receptor), or exhibit a higher permeability for Ca^{2+} than for Na^+ (as the $P2X_4$ receptor where $P_{Ca} / P_{Na} > 4$ for the homomeric channel) [21]. The different P2X receptors not only differ in their pharmacology and ion permeation, but also in their gating properties (for excellent recent reviews see [22, 23]).

1.2.2. P2Y receptors

P2Y receptors, like adenosine receptors, couple to G-proteins that influence the activity of either phospholipase C (PLC) or adenylate cyclase (AC) thus influencing the intracellular production of IP_3 or cAMP, respectively. It is important to note that a given P2Y receptor can couple to different G-proteins in different cell types; therefore, it is always necessary to assess the signalling pathways in the cell under investigation. Nevertheless, $P2Y_{1,2,4,6,11,14}$ receptors

usually activate the IP_3 pathway, while $P2Y_{12,13}$ receptors inhibit AC and thus the production of cAMP (for further details see [13]). It should be noted that P2Y receptors can also, as described for many GPCRs, couple to ion channels [24].

Not only the signalling pathways but also the pharmacology of the P2Y receptors is quite unique [25-28]. Whilst most P2Y receptors can be activated by ATP (the exception being the $P2Y_{14}$ receptor which is activated by UDP-glucose or UDP-galactose); for many ATP is not the primary agonist. $P2Y_{2,4,6}$ receptors are considered as pyrimidine receptors as they are more (or at least as in case of $P2Y_2$) sensitive to UTP or UDP than to ATP. On the other hand, $P2Y_{1,12}$ receptors are sometimes referred to as ADP receptors as ADP is a better agonist (especially for $P2Y_{12}$) than ATP.

1.2.3. Expression profile of P2 receptors

The coupling of the P2 receptors to different intracellular signalling pathways in different cell types is already interesting, but the most intriguing feature of these receptors is their expression pattern. Essentially all cells studied so far seem to express a number of different P2 receptors on their surface membrane resulting in a specific expression pattern characteristic to the given cell [29, 30]. In certain cases, P2X and P2Y receptors can co-exist and modify the effect of one another. This modification can appear as a synergistic effect on $[Ca^{2+}]_i$ (for reviews on certain cell types see [31, 32]), or through the intracellular regulation of the receptor [33]. This expression pattern of P2 receptors changes during differentiation, and the pattern seems to be specific for the stage of differentiation. In skeletal muscle, for example, as we described it in a series of papers [34-36], purinergic signalling is present on embryonic and cultured cells, while it is absent on adult muscle fibres. Furthermore, during differentiation in culture, myoblasts responded with small, smaller myotubes with large, while large, multinucleated myotubes again with low level changes in $[Ca^{2+}]_i$ to purinergic stimulation,

reflecting on the probability of a differentiation state-dependent expression pattern of purinoreceptors [37].

2. Purinergic signalling in skeletal muscle differentiation

Multi-nucleated mature skeletal muscle fibres generally form through the fusion of myoblasts developed from mesodermal progenitors. During embryogenesis, myoblasts can either proliferate, or differentiate into myotubes. Muscle satellite cells located between the basal lamina and the sarcolemma of muscle fibres represent a committed stem cell population that is responsible for the postnatal growth and regeneration of skeletal muscle. Satellite cells possess multi-lineage differentiation potential, and are thus capable of differentiating into osteocytes, adipocytes or myocytes [38, 39]. Skeletal myogenesis depends on the strict regulation of specific gene subsets that control the differentiation of the myogenic progenitors into myofibres. The transcription factors Pax3 and Pax7 play essential roles in the early specification, migration, and myogenic differentiation of satellite cells. Myogenic progenitors expressing Pax3 and Pax7 exhibit increased levels of the myogenic regulatory factors (MRFs) consisting of a group of transcription factors (MyoD, Myf5, myogenin, MRF4) that regulate the skeletal muscle developmental programme. MRF proteins contain a conserved DNA-binding domain that binds the E box sequence CANNTG. Myf5 and MyoD are expressed in proliferating myoblasts and are subject to cell cycle-dependent regulation. Differentiation is then induced upon the activation of myogenin together with MEF2 [40].

Changes in $[Ca^{2+}]_i$ exert a profound influence on muscle differentiation. The Ca^{2+}-calmodulin-dependent Ser/Thr phosphoprotein phosphatase calcineurin (protein phosphatase 2B or PP2B) is one of the molecular targets of cytosolic calcium in the regulation of skeletal muscle differentiation [41]. When activated by Ca^{2+} and calmodulin binding, calcineurin affects gene expression by dephosphorylating specific substrates, including the four

calcineurin-dependent members of the nuclear factor of activated T lymphocytes (NFAT) gene family, NFATc1, NFATc2, NFATc3, and NFATc4. Following dephosphorylation, NFAT translocates from the cytoplasm to the nucleus and activates target genes [42]. Overexpression of the canonical TRPC receptor type 1 (TRPC1) in C2C12 myotubes resulted in altered store operated calcium entry (SOCE), and attenuated differentiation, accompanied by a lower level of nuclear expression of NFAT1 [43]. The neonatal sarcoplasmic/endoplasmic Ca^{2+}-ATPase 1b (SERCA1b) is the major Ca^{2+} pump in myotubes and young muscle fibres. Interference with SERCA1b mRNA expression results in decreased skeletal muscle differentiation via the regulation of $[Ca^{2+}]_i$ [44].

2.1. P1 adenosine receptor expression and function during skeletal muscle differentiation

Differentiation of skeletal muscle fibres both *in vivo* and *in vitro* is accompanied by the development of voltage-dependent processes and alterations in purinergic signalling. Although the role of adenosine in the regulation of blood supply to skeletal muscle is widely discussed, relatively little is known about the role of P1 adenosine receptors in the function of mature skeletal muscle and in myogenesis. The major source of adenosine formation in skeletal muscle is AMP 5'-nucleotidase, which dephosphorylates AMP. In the muscle interstitium, adenosine has been reported to appear as the result of nucleotide degradation within the skeletal muscle fibres and released to the extracellular space in response to contractions [45]. Adenosine receptor transcripts have been identified in skeletal muscle homogenates decades ago; a high expression level of A_{2A} receptor mRNA, moderate signals for A_{2B}, and no signals for A_1 and A_3 receptors have been reported [46]. Later, A_{2A} and A_{2B} receptors were demonstrated in the sarcolemma and cytosol of human skeletal muscle fibres [47]. Skeletal muscle injury elicited a dramatic increase in the expression of A_{2B} and A_3, but not A_1 and A_{2A} adenosine receptors [48]. Moreover, a recent publication suggests a role of A_{2B} receptor-mediated modulation of

the properties of acetylcholine receptors on developing mouse myotubes, resulting in sustained intracellular Ca^{2+} spikes [49]. Based on these important findings, P1 adenosine receptors may become potential targets for stimulation of skeletal muscle tissue regeneration and functional restoration after injury.

2.2. P2 receptor expression and function during skeletal muscle differentiation

The P2 receptor-mediated signal transduction pathway is present on both cultured and freshly isolated embryonic skeletal muscle progenitor cells from mammalian species. $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_4$ receptors have also been demonstrated on multinucleated mouse myoblasts, in addition to $P2X_6$ receptors that have been described in developing rat skeletal muscle [50]. Metabotropic P2Y receptors could also be detected on developing C2C12 mouse myotubes and on embryonic skeletal muscle; furthermore, $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were also shown to be only temporarily expressed in the embryonic developmental stage and down-regulated in postnatal life [36]. Extracellular ATP can regulate myoblast proliferation and the differentiation of mammalian skeletal muscle [51, 52]. During differentiation of C2C12 myoblasts, a continuous increase in their responsiveness to extracellularly applied ATP, as measured by the subsequent change in $[Ca^{2+}]_i$, could be detected [37]. When ATP-evoked changes in $[Ca^{2+}]_i$ were investigated, application of extracellular ATP induced a calcium flux that displayed an early peak and then declined to a steady level. The transient component of calcium flux depended on the activation of P2X receptors and consequent depolarisation, whereas P2Y receptors were found to be responsible for most of the maintained calcium flux. Moreover, the time course of the ATP-evoked flux changed with differentiation [53]. $P2Y_1$ and $P2Y_2$ receptors have been identified in human skeletal muscle and ATP evoked an increase in $[Ca^{2+}]_i$ in human myotubes [35, 54]. GTP has also been shown to play a role in the early phases of skeletal muscle differentiation [55]. Whilst purinergic receptors clearly play significant roles

during the development of skeletal muscle, differentiated muscle fibres barely express them; nonetheless, they reappear after denervation [50].

PKCα overexpression changed the purinergic receptor expression pattern of immature myoblasts and resulted in higher amplitude increases in $[Ca^{2+}]_i$ elicited by ATP, but failed to do so after depolarising stimuli [37]. Whilst nucleotides activate skeletal muscle ryanodine receptors (RyR1), adenosine inhibits the ATP-evoked activation of these receptors. Although ATP reversibly activated RyRs, AMP and adenosine were much weaker agonists [56]. Ca^{2+} influx into C2C12 cells via P2X receptors contributes to the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2); ATP, ADP, 2MeSADP, UTP and UDP induced a transient increase in the level of p42 and p44 phosphorylation [50, 57]. This process was fully Ca^{2+} dependent. On the other hand, ATP activated inositol phosphate formation via a $P2Y_1$ receptor-dependent manner in primary human skeletal muscle cells. In addition, ATP elicited ERK1/2 phosphorylation in a time and concentration dependent manner, also mainly via $P2Y_1$ receptors. The ATP mediated ERK1/2 phosphorylation was strictly dependent on PLC and phosphoinositide 3-kinase (PI3-K) activity [58]. The involvement of purinergic receptors in skeletal myogenesis is summarised in Fig. 2.

3. Ion channels and Ca^{2+} signalling of developing chondrocytes

3.1. Major characteristics of embryonic cartilage formation

Hyaline cartilage plays a very important role during the formation of vertebrate skeletal system as it provides the primitive skeleton of embryos and serves for templates of endochondral bone formation. Cartilage develops from mesenchymal tissue in the process called chondrogenesis, which is characterised and governed by the expression and activity of the master transcription factor Sox9 [59]. Pluripotent mesenchymal cells proliferate and migrate intensely to form precartilage condensations of chondrogenic cells, in which

intercellular junctions and local paracrine signalling pathways drive chondroprogenitors towards their final commitment (for reviews see [60, 61]). Initially elongated, the chondroprogenitor cells undergo characteristic morphological changes when they turn into matrix-producing young chondrocytes, obtaining the biomechanically optimal round shape (Fig. 3). Transformation of the macromolecular composition of the extracellular matrix (ECM) and adhesion of the cells to the altered microenvironment have fundamental influence on chondrogenesis [62]. Ultimately, terminal differentiation resulting in hypertrophic chondrocytes, calcification of cartilage ECM, and invasion of blood vessels precedes bone formation. In every step of cartilage formation, many players of the signalling pathways are influenced by local or generalised changes of intracellular Ca^{2+} concentration [63].

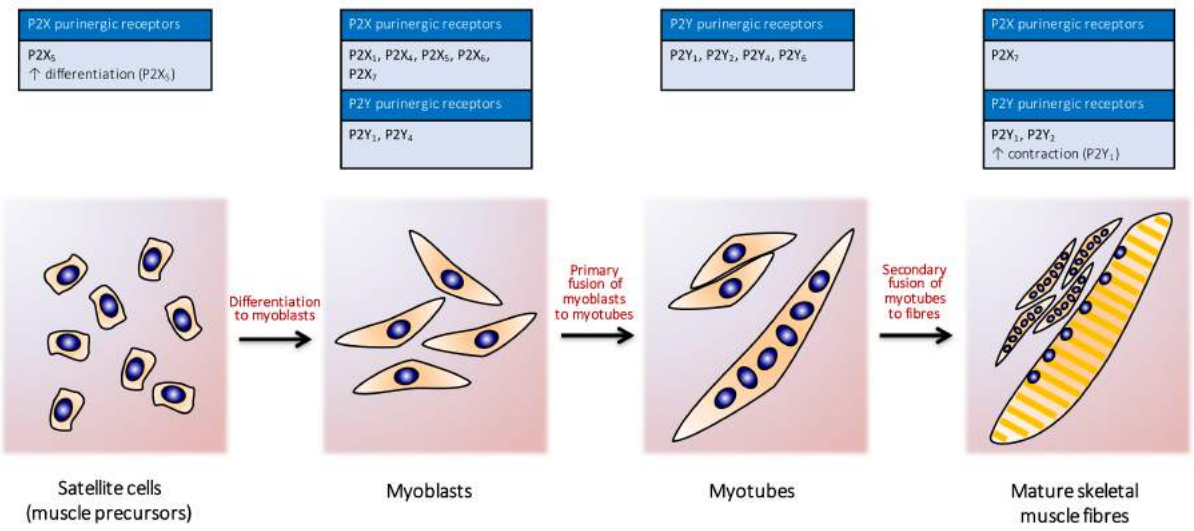


Figure 2. Mammalian skeletal myogenesis. Myogenic precursor cells (also known as satellite cells in adults) start to proliferate and differentiate into myoblasts. These mononuclear cells proliferate and fuse together (primary fusion step) to give rise to multinucleated myotubes. Additional myoblasts fuse to the existing myotubes in the secondary fusion step to produce even larger myotubes which eventually align to form muscle fibres. This process is mediated by P2X and P2Y receptors. Where data are available, upwards arrows indicate increased activity of downstream pathways following receptor activation. For further details, please see text.

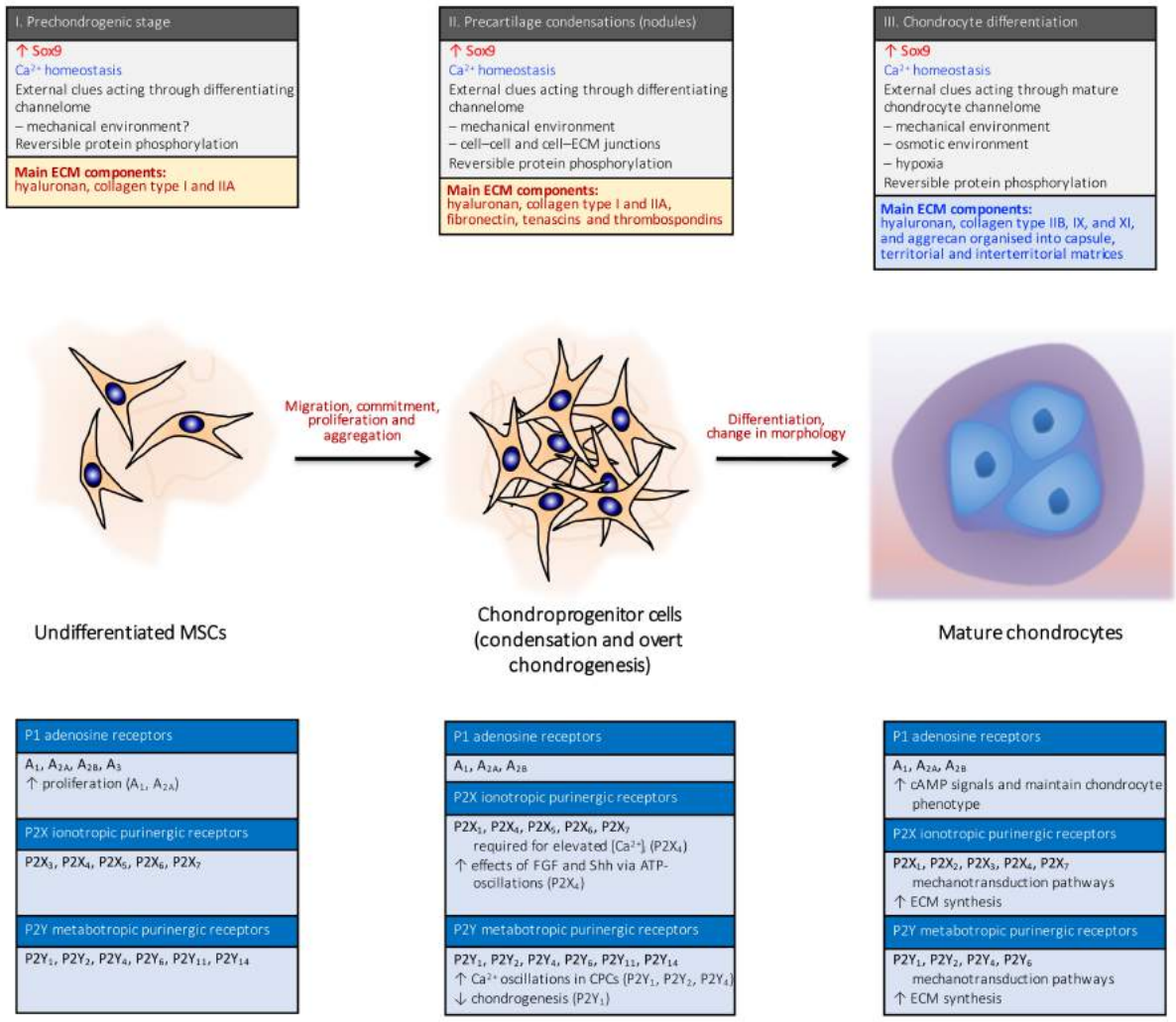


Figure 3. The main steps of chondrogenesis. Undifferentiated mesenchymal stem cells (MSCs) are recruited to the sites of cartilage formation. Following migration and local proliferation cell density increases (condensation), which triggers a set of intracellular events culminating in overt chondrogenesis accompanied by a change in morphology (acquire the characteristic ovoid shape) and start to deposit cartilage ECM molecules. Amongst other factors, the process is regulated by the master chondrogenic transcription factor Sox9, which is responsible for the expression of ECM components. The precisely set Ca^{2+} homeostasis and the tight regulation of other ionic currents through components of the channelome of differentiating and mature chondrocytes are essential in mediating the effects of external cues of the chondrocyte niche such as the mechanical or osmotic environment. Various protein kinases/phosphoprotein phosphatases also play essential roles in the control of chondrogenesis. The involvement of P1 adenosine receptors, as well as P2X and P2Y purinergic receptors in modulating the main steps of chondrogenesis is shown in boxes below the schematic diagrams. Where data are available, arrows indicate increased (upwards arrow) or decreased (downwards arrow) activity of downstream pathways following receptor activation. See further explanation in text.

Permanent hyaline cartilage remains present at certain locations of the skeleton, such as the nasal septum, the wall of airways and the surfaces of bones in synovial joints. Articular cartilage differs from other permanent cartilages by the lack of perichondrium on its surface. This feature is an important factor in the very low regeneration capability of this tissue. Hyaline cartilage is unique among connective tissues in several aspects: it is colonized by a single cell type, the chondrocyte; it does not contain nerves; and healthy cartilage is not penetrated by blood or lymphatic vessels [64]. Mature chondrocytes divide rarely if at all and live in a unique niche in a specialised ECM, which is the product of these cells. A typical location of this tissue in the skeletal system is the surface of bones in synovial joints, where complex load, deformation, and fluid shear are exerted on articular cartilage during locomotion and other physical activities. Chondrocytes are also challenged by osmotic and hypoxic stress under physiological circumstances [65]. Whilst optimal mechanical stimulation is necessary for cartilage development [66] and homeostasis, extreme levels of loading have also been shown to initiate cartilage degradation [65]. A recent publication reported that *in ovo* immobilization of chicken embryos resulted in fused articular surfaces in the knee joint [67]. Albeit chondrocytes are generally considered as non-excitable cells the above findings all suggest that either mature or differentiating chondrocytes are able to "sense" various environmental stimuli and transmit those into the cells, where signal transduction pathways influencing metabolism, transcriptional activity, differentiation programmes or proliferation of cartilage cells can be altered.

3.2. Ion channels and intracellular Ca^{2+}-concentration changes in chondrogenic cells

Plasma membrane ion channels are the primary candidates to link these extracellular stimuli to the internal milieu of either developing or mature chondrocytes. Indeed, various

plasma membrane receptors which are sensitive to mechanical load, fluid sheer or osmotic changes, are present and function on mature chondrocytes (for a review, see [68]). Some of them, such as several members of the Transient Receptor Potential (TRP) channel family [69], purinoreceptors [70], or the *N*-methyl-D-aspartate type ionotropic glutamate receptor (NMDAR) [71] are known to function as cation channels with considerable permeability for Ca^{2+} and are involved in the Ca^{2+} homeostasis of pluripotent mesenchymal stem cells (MSCs) (for a recent review, see [63]).

Cartilage formation requires a concerted action of numerous intracellular signal transduction molecules [60], many of which are sensitive to the changes of intracellular Ca^{2+} concentration. Our previous findings indicated that inhibition of calcineurin by cyclosporine A seriously impaired *in vitro* chondrogenesis in chicken limb-bud derived high density cultures (HDC) [72]. We also detected precisely set changes in basal cytosolic Ca^{2+} concentration during chondrogenesis when a day-by-day variation of this cellular parameter was detected by single cell fluorescent Ca^{2+} imaging [73]. A characteristic peak in cytosolic free Ca^{2+} concentration was found on day 3 of culturing (approximately 140 nM, as compared to 100 nM prior to or after this day). Of note, this is the period of the culturing when the majority of the chondrogenic cells differentiate to matrix producing chondroblasts in this model. This peak in cytosolic Ca^{2+} concentration was found to be indispensable to proper differentiation of cells in HDC because chelation of extracellular Ca^{2+} on culturing days 2 or 3 resulted in significantly lower cytosolic Ca^{2+} concentration and impaired chondrogenesis, demonstrated by an almost completely abolished cartilage matrix production. In these experiments, proliferation of the differentiating chondrogenic cells was extremely dependent on a well-defined range of cytosolic Ca^{2+} concentration [73]. In further experiments, we have proved the dependence of the above changes in cytosolic Ca^{2+} concentration on purinergic signalling via ionotropic $P2X_4$ receptors [74]. Besides the long term day-by-day changes of the cytosolic Ca^{2+} concentration,

we have also detected rapid Ca^{2+} oscillations in the differentiating chondrocytes. The periodicity of these oscillations was approximately 4–5 transients/minute and the oscillations in chondrifying mesenchymal cells were found to be mainly dependent on the availability of extracellular Ca^{2+} as removal of free Ca^{2+} from the bath solution immediately abolished these oscillations in most of the cells examined. However, store-operated calcium entry (SOCE) was also found to be essential for the maintenance of the Ca^{2+} oscillations [75]. We demonstrated that a developmental switch in the expression and function of voltage-gated potassium channels [76] and activity of voltage dependent Ca^{2+} channels [75, 77] influenced Ca^{2+} oscillations in chondrogenic cells.

The significance of Ca^{2+} signalling in chondrogenesis can be underlined by the fact that Sox9, the master transcription factor of chondrogenesis and key element of sex determination, can be translocated to the nucleus either via calmodulin in a Ca^{2+}-dependent fashion or via importin-β in a Ca^{2+}-independent mechanism. Impairment of the calmodulin-driven nuclear shuttle of Sox9 causes campomelic dysplasia/sex reversal, a severe human syndrome affecting skeletogenesis and sexual differentiation [78].

Considering the above, the importance of the changes of intracellular Ca^{2+} concentration during differentiation of chondrocytes, as well as in maintenance of the healthy mature cartilage seems to be unquestionable. In the following sections of this review article, we focus on the current knowledge on the significance of purinergic signalling in the regulation of the differentiation and Ca^{2+} homeostasis of chondrocytes

4. Purinergic signalling during chondrogenesis

Mature articular chondrocytes have been shown to express various P1 and P2 purinergic receptors including A_1, A_{2A} and A_{2B}, as well as different subtypes of P2X ($P2X_{1,2,3,4,7}$) and P2Y ($P2Y_{1,2,4,6}$) receptors [79, 80]. Given that P1 and P2 receptors are important regulators of

mechanosensation or mechanotransduction in mature chondrocytes [79], it is plausible to assume that they may also be involved in the regulation of chondrogenesis.

The role of purinergic signalling has been widely studied in the differentiation of many cell types (for a review, see [81]). Since the development of cartilage (and to a limited extent its repair) is dependent on the differentiation of chondroblasts and chondrocytes from chondroprogenitor MSCs [82], we will focus on the chondrogenic differentiation of these cells in the context of purinergic signalling. Despite the limited number of studies in this field, accumulating evidence supports the importance of extracellular nucleotides in the regulation of maintenance, proliferation and differentiation of MSCs.

4.1. P1 adenosine receptor expression and function during chondrogenesis

As it could be expected from a cell type with a broad, multilineage differentiation potential, MSCs were found to express $A_{1,2A,2B}$ and A_3 receptor subtypes of the P1 adenosine receptor family; activation of A_1 and A_{2A} receptors has been shown to increase proliferation, whilst the most abundant A_{2B} receptors mediate osteogenic differentiation of primary human bone marrow stromal cells [83]. In a different study, stimulation of the A_1 receptor was demonstrated to enhance osteogenic differentiation of human dental pulp-derived MSCs [84]. Interestingly, no data are available as to whether P1 adenosine receptors are *de facto* involved in the regulation of chondrogenesis. In a migratory cell population derived from human osteoarthritic knee articular cartilage with a strong chondrogenic differentiation potential termed CPCs [85], we have recently established the P1 purinergic receptor profile, which correlated well with that of MSCs as CPCs expressed A_1, A_{2A}, and A_{2B}, but no A_3 receptors [86]. It should be noted that the same P1 receptor subtypes were found to be expressed in articular chondrocytes [87]. A_{2A} and A_{2B} adenosine receptors are of particular importance as these are coupled to G_s, which stimulates AC and produces a cAMP signal [88]; cAMP

mediated signalling is a well known mediator of chondrogenesis as well as in maintaining the chondrocyte phenotype [89].

4.2. P2 purinergic receptor expression and function during chondrogenesis

Compared to P1 receptors, more data supporting the role of P2 receptors in chondrogenesis is available. However, there are some controversies as to whether purinergic signalling is a positive or negative regulator of chondrogenesis; some reports suggest that extracellular nucleotides negatively regulate cartilage metabolism whilst others describe beneficial effects of ATP.

The P2 receptor profiling of human MSCs derived from various sources have only recently been performed. Adipose tissue-derived stem cells (ATSCs) and dental follicle cells (DFCs) derived from the ectomesenchyme expressed some P2X ($P2X_{3,4,5,6,7}$) and all 8 P2Y receptor subtypes; interestingly, no mRNA transcripts of $P2X_1$ and $P2X_2$ receptors were identified in either cell type [90, 91]. Extracellular nucleotides acting through P2 receptors (primarily $P2X_6$, $P2Y_4$, $P2Y_6$, $P2Y_{13}$ and $P2Y_{14}$) are important modulators of MSC differentiation towards the osteogenic and adipogenic lineages [90-92]. The purinergic concept of MSC differentiation is further supported by a recent study showing substantial changes in ecto-nucleotide metabolism and ecto-nucleotidase expression profiles following chondrogenic differentiation of human umbilical cord-derived MSCs [93].

The specific mechanism as to how P2 receptors mediate these events remains elusive. Ca^{2+} signalling is a major regulator of differentiation [63] and nucleotides acting through P2 receptors can either elicit Ca^{2+} influx or mobilise Ca^{2+} from intracellular stores. The Ca^{2+} homeostasis of MSCs has been mapped earlier and Ca^{2+} oscillations have been reported to be driven by an autocrine/paracrine purinergic loop, whereby ATP released through hemi-gap junctions triggered $P2Y_1$ metabotropic purinergic receptors and Ca^{2+} release via PLC-β and

IP_3R signalling [94]. In migratory CPCs, we have recently confirmed the mRNA and protein level expression of certain P2X ($P2X_{1,4,5,6,7}$, but not $P2X_2$ and $P2X_3$) and all P2Y receptors [86]. Our results suggest the functionality of $P2Y_1$, $P2Y_2$, $P2Y_4$, as well as $P2X_4$ and $P2X_6$ receptor subtypes in CPCs, which shows a very good correlation with previous data implicating the P2 receptor subtypes $P2X_6$, $P2Y_4$ and $P2Y_{14}$ to be key regulators in MSC commitment [91]. Given the multilineage differentiation capacity of CPCs to the osteogenic, adipogenic and chondrogenic lineages, their P1 and P2 receptor profiles are in good correlation with the above data. Further experiments need to confirm the specific role of these receptors in lineage-specific differentiation.

Whilst signalling through P2 receptors seems to promote differentiation of MSCs, their role in chondrogenic micromass cultures is less straightforward. The first study on the involvement of purinergic signalling in chondrogenesis *in vitro* showed that ATP acting on the $P2Y_1$ receptor inhibited cartilage formation in HDCs of embryonic chick limb bud-derived mesenchymal cells [95]. In contrast, our group has reported that cells in HDC responded to extracellular ATP by elevating their intracellular Ca^{2+} levels mainly at the time of chondroblast formation and administration of ATP to the culture medium stimulated chondrogenesis [74]. Whilst Western blot analyses revealed the presence of many purinoreceptors, i.e. $P2X_{1,4,5}$ and $P2X_7$ of the P2X-family and $P2Y_{1,2,3,4}$ and $P2Y_5$ of P2Y receptors in cells of differentiating micromass cultures, the receptor primarily responsible for the rise of Ca^{2+} concentration evoked by extracellular ATP seemed to be $P2X_4$ receptor [74]. Earlier we reported that this receptor contributed to the elevation of cytosolic Ca^{2+} levels of chondrogenic cells on day 3 of culturing [73]. The apparent discrepancy between these studies may be explained by the fact that whilst Meyer and colleagues used cultures established from mesenchymal cells derived from the forelimbs only [95], the micromass cultures used in our study were of mixed origin containing progenitor cells from both fore and hind limb buds [74]. It has long been known

that chondrogenesis in HDC derived from wing and leg precartilage cells is affected differently by certain morphogens such as transforming growth factor-β1 (TGF-β1) or retinoic acid, and they also differ from each other in condensation morphology and organisation of fibronectin during the early phase of differentiation [96]. The discrepancy may also indicate that ATP, acting through different purinergic receptor subclasses, is able to precisely, albeit sometimes contrarily, regulate chondrogenesis.

Another study has also shown that intracellular oscillations in ATP, acting via $P2X_4$ receptors, have an essential role in pre-chondrogenic condensation [97] and mediate the inductive action of fibroblast growth factor (FGF) and sonic hedgehog (Shh), proposing the hypothesis that morphogens organise skeletal patterns via ATP oscillations [98]. Purinergic signalling was reported playing role in foetal articular cartilage repair in rat embryos *in vivo*. Although the authors did not identify the specific receptor involved in the process, they showed the presence of a signalling pathway that used the intercellular transfer of ATP and increased cytosolic Ca^{2+} levels, and expression of *c-fos* in foetal articular cartilage [99]. The involvement of P1 and P2 receptors in chondrogenesis are summarised in Fig. 3.

5. Concluding remarks

It is clear that precisely set intracellular Ca^{2+}-concentration changes exhibited by developing chondrocytes and skeletal muscle fibres play essential roles at every step of their differentiation. Purinergic signalling is one of the most ancient mediators of cell-to-cell communication and can modulate the pattern of the intracellular Ca^{2+}-concentration changes. Bone, skeletal muscle and cartilage abundantly express various purinergic receptors and accumulating evidence support that extracellular purines participate in the regulation of the proliferation, differentiation and mechanosensation of skeletal tissues. Developing chondrocytes and skeletal muscle fibres express various types of purinoreceptors, offering the

ability of their responsiveness to ectopurines and experiments prove that both tissues contain resident cells secreting various purinergic ligands.

On the basis of these experimental data, one can propose that it would be plausible to utilize ectopurines to facilitate tissue regeneration of damaged cartilage or skeletal muscle. Nonetheless, the ubiquitous nature of signalling mediated by ectopurines, that is, its presence and operation in virtually all tissues and cells, has to be considered if any *in vivo* therapeutic intervention is designed to target this mechanism.

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Contributors

The authors researched, discussed and approved the concept, drafted and submitted the commissioned paper. All co-authors made a significant intellectual contribution to the concept of the manuscript.

Conflict of interest statement

The authors wrote this paper within the scope of their academic and affiliated research positions. The authors do not have any commercial relationships that could be construed as biased or inappropriate. There are no competing interests.

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