[Frontiers in Bioscience, Scholar, 5, 305-324, January 1, 2013]

Calcium signalling in chondrogenesis: implications for cartilage repair

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1. ABSTRACT

Undifferentiated mesenchymal stem cells (MSCs) represent an important source for cell-based tissue regeneration techniques that require differentiation towards specific lineages, including chondrocytes. Chondrogenesis, the process by which committed mesenchymal cells differentiate into chondrocytes, is controlled by complex but not yet completely understood mechanisms that involve many components, including intracellular signalling pathways, as well as plasma membrane receptors and ion channels. Some of these signalling components are Ca^{2+} sensitive. Although the Ca^{2+}-signalling toolkit of undifferentiated MSCs and mature chondrocytes are extensively studied, the adaptation of these components during differentiation and their role in chondrogenesis is not adequately established. In this review, various aspects of Ca^{2+} signalling are discussed in MSCs and in mature chondrocytes including spatial and temporal aspects, as well as Ca^{2+} entry and elimination processes, with implications for their involvement in chondrogenesis. A better understanding of these pathways is envisaged to provide a more efficient differentiation of MSCs towards chondrocytes that may lead to the development of better cartilage tissue engineering techniques.

2. INTRODUCTION

Prevalence of musculoskeletal disorders (comprised more than 100 conditions, including various rheumatic, arthritic and joint diseases) is constantly increasing owing to unfavourable changes in the population of developed countries, exerting an ever-growing burden on healthcare systems around the globe (1). Osteoarthritis (OA), characterised by inflammation and breakdown of the shock-absorbing articular cartilage within the joint, is the most common form of chronic musculoskeletal disorders. However, no effective or curative treatment is available for OA. Therefore, there is a pressing socio-economic need for the development of novel and innovative therapeutic strategies to preserve or regenerate the natural articular cartilage and its underlying bone. During the last decade, with an increase in our knowledge about the molecular and biological characteristics of embryonic or adult pluripotent mesenchymal stem cells (MSCs), their applicability in regenerative medicine has emerged. However, regeneration of proper articular cartilage is still a challenge, mainly due to the fact that albeit extensive research has been conducted in this field over the last 50 years, our current knowledge of the molecular steps and precise regulation of chondrogenesis is far from being complete.

In this review, after a brief overview of cartilage biology and the normal course of chondrogenesis, we will focus on Ca^{2+} dependent signalling pathways that regulate this process; in particular, Ca^{2+} signalling in MSCs and adult articular chondrocytes will be described, with implications for the involvement of these pathways in chondrogenesis and possible cartilage regeneration techniques.

3. ADULT MESENCHYMAL STEM CELLS IN CARTILAGE REPAIR

Originally isolated from bone marrow by Pittenger and colleagues in 1999 (2), somatic or adult MSCs have been described to differentiate into various connective tissue cells, including the chondrogenic lineage that can give rise to chondroblasts and chondrocytes (3). MSCs have been identified in various mature tissues, including skeletal tissues, such as bone and cartilage (4). Indeed, the intrinsic regeneration capacity of cartilage that is exploited during the treatment of early-stage osteoarthritic patients (*e.g.* Pridie-drilling, microfracture, etc.) can mainly be accounted for the cartilage-forming capacity of bone marrow-derived MSCs (5, 6). However, it is fibrous cartilage, rather than hyaline cartilage, that is formed *in situ* as a result of such bone marrow-opening techniques, which is characterised by less favourable biomechanical properties.

Owing to the apparent insufficiency of physiological repair mechanisms to regenerate the proper structure of articular hyaline cartilage, the concept of cell-based therapies has emerged. MSCs represent an ideal source of these cells, due to their capacity to differentiate into chondrocytes (7). MSC-based cartilage regeneration strategies hold much potential for treating large-scale lesions as the currently accepted and clinically used autologous chondrocyte implantation (ACI) techniques can only be successfully applied to treat smaller cartilage lesions (8). However, to overcome the apparent difficulties of articular cartilage regeneration, a better understanding of signalling mechanisms that govern chondrogenesis of MSCs is required.

4. ARTICULAR CARTILAGE: FROM STRUCTURE TO FUNCTION

For attempts in articular cartilage tissue regeneration to be eventually successful, an in-depth knowledge of the structure, components, as well as biomechanical characteristics of the mature tissue is essential. Hyaline cartilage provides the source of bones formed by endochondral ossification; enables bone elongation at epiphyseal growth plates; covers the articular surfaces of joints; and supports the wall of various internal organs (9). Articular cartilage is a special kind of hyaline cartilage that essentially comprises two main components: chondrocytes, the single cell type of cartilage, are located in cavities called lacunae and are embedded in a unique extracellular matrix (ECM). In hyaline cartilage, 3–4 chondrocytes usually form small clusters known as isogenous cell groups (10). Adult articular chondrocytes in mature articular cartilage are enigmatic cells: first, they are postmitotic cells, thus considered as immortalised cells that maintain the proper composition of cartilage ECM throughout a lifespan; second, they are able to withstand enormously high compressive forces in major weight bearing joints; third, due to lack of blood vessels in cartilage, these cells can produce ATP required for their metabolic functions by obligate anaerobic respiration and are able to synthesise large amounts of ECM components in spite of low oxygen tension and nutrient supply; and fourth, they are able to survive the harsh conditions (*i.e.*, the low extracellular pH owing to lactate accumulation) brought about in part by their own metabolic activities (11). The fact that mature chondrocytes are postmitotic cells can explain why articular cartilage possesses a limited healing response to various injuries.

The cell-to-ECM ratio in articular cartilage is approximately 1 to 10 by volume, which refers to the importance of proper matrix composition in cartilage function (12). Cartilage ECM consists of four main molecule groups: collagens, proteoglycans (PGs), glycosaminoglycans (GAGs) and non-collagenous glycoproteins. The cartilage-specific collagen type II accounts for more than 80% of all collagens present in the matrix. The primary function of collagen type II fibrils is to provide tensile strength; moreover, its highly regular arrangement can be accounted for the zonal architecture of articular cartilage (13). Collagen type IX binds other matrix components to the collagen network; collagen type XI adjusts the length of ECM fibres; and collagen type VI is an essential component in binding chondrocytes to the matrix (14).

PGs that form a gel-like ground substance are also essential ECM components; they provide stability and durability during compressive load. Aggrecan is the most characteristic cartilage-specific PG. Aggrecan monomers consist of chondroitin-sulphate and keratan-sulphate side chains bound to a core protein; by connecting to hyaluronic acid, one of the fundamental cartilage GAGs, the monomers form huge PG aggregates. By having a very high negative charge, glycosaminoglycan side chains can strongly bind to water. The water content of the ECM is of crucial importance: it provides the milieu for diffusion of oxygen and nutrients. This is especially important when considering that chondrocytes can be millimetres away from the nearest blood vessel. Together with GAGs, water also provides cartilage with characteristics similar to a shock absorber: under compressive load water flows out and then it flows back in, bringing about the structural basis of elasticity (15). On account of the high negative charge of PGs, chondrocytes are exposed to an unusual ionic environment because GAG side chains attract large numbers of cations, such as Na^{+}, K^{+} and Ca^{2+} ions, creating a high extracellular osmolarity and probably influencing the resting membrane potential of these cells (11, 16). Non-collagenous glycoproteins can either connect chondrocytes to the matrix (*e.g.* tenascin, fibronectin, chondronectin or anchorin CII) or provide connections between matrix molecules, including matrilin-1 and link protein (17).

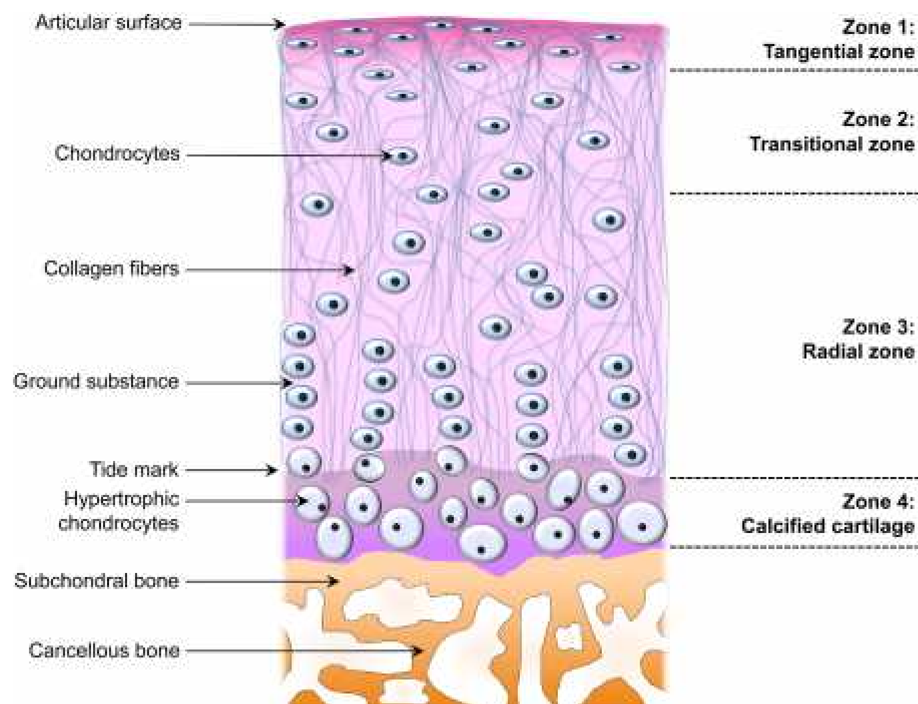

Figure 1. Zonal architecture and orientation of collagen type II fibers in mature articular cartilage. Each zone is characterized by different morphology and function of chondrocytes and specific, arcade-like orientation of collagen fibers: in the superficial zone (zone 1), the orientation of these fibers is horizontal and chondrocytes in this zone secrete a proteoglycan-rich molecule called lubricin (also known as PRG4), a lubricant for the articular surface; in zone 2, collagen fibers run obliquely; and in the radial zone, their orientation is perpendicular to the articular surface. The boundary between non-calcified and calcified matrix is the tide mark where the proteoglycan content of the matrix is reduced; below this area, hypertrophic chondrocytes are present and significant amount of type X collagen appears along with calcification of the ECM. The zonal architecture is indispensable to proper biomechanical characteristics of articular cartilage.

Ultrastructurally, it is the high level of organisation of major ECM components (mainly that of collagen type II fibres) that clearly distinguishes mature articular cartilage from other types of hyaline cartilage. The orientation of collagen type II fibres, slight changes in ECM composition, as well as the morphology and function of chondrocytes bring about 4 distinct zones in articular cartilage (1) the superficial or tangential zone, (2) the middle (or transitional) zone, (3) the deep (or radial) zone, and (4) the zone of calcified cartilage (Figure 1) (for a review, see (18)). Obviously, the superficial zone of articular cartilage is a critical component of the mature tissue because its collagen fibrils are oriented parallel to the surface, providing resistance to shear forces in the joint. Chondrocytes in this zone were also proved to be characterized by unique gene expression profiles: they secrete lubricin, a glycoprotein-like molecule reported to provide lubrication for articular surfaces, and its role in various joint diseases (rheumatoid arthritis and osteoarthritis) has also been implicated (19). Moreover, several studies suggest that articular cartilage has a different source than epiphyseal cartilage: in the rabbit, cartilage lining the ends of bones at birth is replaced by bone and cartilage of the articular surfaces is formed anew at the beginning of post-natal life by appositional growth (13). In accordance with this concept, cells with stem cell-like properties were isolated from the tangential zone of developing articular cartilage in mice and cattle (20). Notwithstanding these preliminary results concerning the development of the superficial zone of articular cartilage, a deeper understanding of the establishment of the zonal architecture of mature articular cartilage is required in order to create bioengineered cartilage with appropriate biomechanical properties.

5. CHONDROGENESIS IS REGULATED BY INTERPLAY BETWEEN NUMEROUS INTRA- AND EXTRACELLULAR FACTORS

Cartilage appears prior to bone formation during skeletal development; it is derived from the undifferentiated embryonic connective tissue (mesenchyme) during a process that starts with the condensation and nodule formation of chondroprogenitor cells. Proper cellular density is a crucial factor at the initial step of either *in vivo* or *in vitro* cartilage formation; consequently, the majority of *in vitro* chondrogenesis models apply unusually high number of cells (more than 15 M chondrogenic cells/mL) to mimic condensation and these models are therefore known as micromass or high density cell cultures (HDC) (21, 22). When chondroprogenitor cells isolated from limb buds of chicken (or less frequently mouse) embryos are used to establish HDC, this system is referred to as a primary

chondrogenesis cell culture model. A great advantage of this model is the inherent ability to form hyaline cartilage within one week. Data of our own laboratory discussed here were gained from experiments using chicken HDC.

Besides transient appearance of Ca^{2+} dependent intercellular junctions in precartilage nodules (N-CAM and N-cadherin; (23)), the differentiation of chondroprogenitor cells into chondroblasts is governed by a number of growth factors and other signal molecules, including fibroblast growth factor (FGF), transforming growth factor-beta (TGF-beta), bone morphogenic protein (BMP) and Wnt families; (24). Cells in precartilaginous aggregates change morphology, and the resultant round-shaped phenotype seems to be essential for proper chondrogenesis, as inappropriate culture conditions of isolated chondrocytes had led to loss of round cellular morphology and a consequent failure to secrete cartilage-specific ECM (25). Changes in the phenotype of chondroprogenitor cells is also associated with altered gene expression pattern; in particular, expression of the chondrocyte-specific transcription factor Sox9 markedly increases, which is regulated by members of the FGF, TGF-beta, BMP and Wnt families (26). Inevitably, the expression of Sox9 in committed osteochondroprogenitor mesenchymal cells during condensation is the first step towards chondrogenesis to actually take place. Sox9 is responsible for the expression of two other Sox transcription factors, Sox5 and Sox6, and these three Sox transcription factors (often referred to as the Sox trio) in turn control the expression of a number of ECM macromolecules, including collagen types II, VI, IX and XI, CD-RAP (cartilage-derived retinoic acid-sensitive protein) and aggrecan (for a detailed review, see (27)). As a consequence, the ECM surrounding the differentiating chondrogenic cells is also subject to profound changes: cartilage-specific matrix components, most importantly collagen type II and aggrecan are laid down soon after their differentiation (28). Noteworthy that there is a reciprocal interplay between chondrocytes and cartilage-specific ECM: synthesised by chondrocytes, the characteristic composition and organization of ECM is pivotal for maintaining the appropriate morphology and function of chondrocytes (29).

In-depth identification of major intracellular signalling pathways that govern the molecular steps controlling chondrogenesis is essential to be able to externally influence cartilage formation. Mitogen-activated protein kinases (MAPKs) are important components of chondrocyte signalling involved in the conversion of extracellular stimuli into cellular responses and thus coordinate proliferation, differentiation and gene expression. The involvement of MAPKs in chondrogenesis is the subject of several review articles (for details, see (30)) and is only briefly discussed here. The three MAPK pathways contribute to the regulation of chondrogenesis to a various extent: while JNKs seem not to be involved in this process, p38s and ERKs are key regulators of chondrogenic differentiation; in chicken HDC, an opposing role of p38 and ERK has been reported: while the p38 MAPK pathway promotes, activation of the MEK/ERK pathway represses *in vitro* chondrogenesis (31). The results of our laboratory also confirmed the negative role of the ERK1/2 pathway in the regulation of *in vitro* chondrogenesis in the same model (22, 32).

Little is known about the upstream regulators of the MAPK pathways in chondrocytes. As far as the extracellular regulators are concerned, members of the TGF-beta and FGF families, retinoic acid, and integrins are reported to differentially activate p38 and ERK MAPK pathways (30). By considering that the MAPKs relay extracellular stimuli to cellular responses it is not surprising that these pathways are also implicated in mechanotransduction in chondrogenic cells: proliferation and differentiation, via altered MAPK activity, was also found to be influenced by mechanical stimuli during chondrogenesis (30, 33).

Transient protein phosphorylation is the most common posttranslational protein modification, which influences activity of many signalling proteins. Among the intracellular factors that regulate chondrogenesis various Ser/Thr protein kinases and Ser/Thr phosphoprotein phosphatases (PPs) were identified. In chicken HDC, expression of various protein kinase C (PKC) isoforms (PKC alpha, gamma, epsilon, zeta, lambda and iota) was reported and they were found to be mostly required at the early stages of *in vitro* chondrogenesis (34). Both PKC alpha (35) and PKC delta (32) were found to be positive regulators of chondrogenesis. In the same experimental model, the cAMP-dependent protein kinase A (PKA) is reported to be required for precartilage nodule formation by regulating N-cadherin expression and exerts its role via modulation of PKC alpha activity (36). Our laboratory has investigated the involvement of PP1 and PP2A in this process and found that PP2A was a negative regulator of *in vitro* chondrogenesis as administration of its inhibitor, okadaic acid, significantly enhanced cartilage matrix production; moreover, it also resulted in elevated PKA enzyme activity levels (37, 38). To the contrary, the Ca^{2+}-sensitive PP2B (also referred to as calcineurin) was reported to positively control chondrogenesis and to be involved in the regulation of ERK1/2 activity (22). Signalling pathways that govern chondrogenesis in various chondrogenic models are summarised in Figure 2.

Many of the signalling components outlined above (*e.g.* PKC, PP2B) are at least partially calcium-sensitive; activity of the MEK/ERK pathway can also be modulated by Ca^{2+}-calmodulin (39). Appropriate change (amplitude, frequency) in cytosolic Ca^{2+} concentration is often a prerequisite for proliferation or differentiation to take place. Consequently, Ca^{2+} sensitive signalling pathways may play an indispensable role also in chondrogenesis.

6. CALCIUM SIGNALLING: A SINGLE MESSENGER WITH DIVERSE FUNCTIONS

Calcium (Ca^{2+}) is among the most ancient second messengers of eukaryotic cells and is involved in the regulation of various physiological and biochemical processes including secretion, muscle contraction,

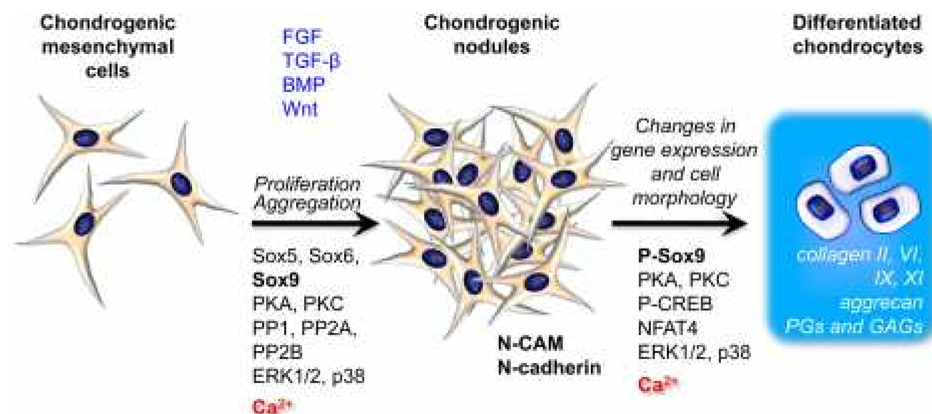

Figure 2. Main steps of chondrogenesis and the major signalling pathways involved in the regulation of this pathway. Initially, chondroprogenitor cells are loosely arranged cells with typical elongated morphology. During condensation, extensive proliferation and migration of chondrogenic cells occurs, resulting in formation of precartilaginous nodules. Soluble factors (such as FGF, BMPs, Wnt) and cell adhesion molecules (N-CAM and N-cadherin) are required for this step. After the differentiation process has occurred, cells gain round morphology and a gene expression pattern required for synthesis of cartilage-specific ECM is also activated. In each step of chondrogenesis, calcium is essential for the function of various signalling pathways. For references please see text. (FGF: fibroblast growth factor; TGF-beta: transforming growth factor beta; BMP: bone morphogenic protein; PKA, PKC: protein kinase A and C; PP1, PP2A and PP2B: phosphoprotein phosphatase 1, 2A and 2B; ERK1/2: extracellular signal regulated kinase; CREB: cAMP-responsive element binding protein; NFAT: nuclear factor of activated T-lymphocytes; PG: proteoglycan; GAG: glycosaminoglycan)

metabolic processes, gene expression, cell division and apoptosis (40). In the extracellular space, Ca^{2+} concentration is in the range of 10^{-3} M; in contrast, Ca^{2+} concentration in the cytosol is significantly lower, generally by four orders of magnitude (~10^{-7} M). Rise in cytosolic Ca^{2+} can either be the result of Ca^{2+} influx from the extracellular space or release from certain cell organelles, including the endoplasmic reticulum (ER) or the mitochondrium (referred to as Ca^{2+} stores). The large electrochemical gradient between these compartments enables the flux of Ca^{2+} ions into the cytosol across Ca^{2+} channels located in either the plasma membrane or in the membrane of the ER or mitochondria. Eventually, the increased cytosolic Ca^{2+} concentration, via numerous effector molecules, evokes the characteristic cellular response, which is terminated by restoring the basal cytosolic Ca^{2+} concentration by active Ca^{2+} pump or exchange mechanisms (41).

The Ca^{2+} signal is precisely regulated both temporally and spatially. The versatility of Ca^{2+} dependent signalling pathways lies in the target proteins with the help of which Ca^{2+} can exert its functions. All proteins that are involved in the regulation and maintenance of Ca^{2+} homeostasis in cells can be considered as members of a global Ca^{2+}-signalling toolkit; all cells express and utilise the most appropriate members of the toolkit that are best required for their proper function (42). Members of the Ca^{2+}-signalling toolkit include plasma membrane receptors, G-proteins, voltage- and ligand-gated Ca^{2+} channels, receptors of the endoplasmic reticulum (inositol-1,4,5-trisphosphate receptor [IP_3R], ryanodine receptor [RyR]), chaperons (calsequestrin, calreticulin), Ca^{2+} pumps (plasma membrane Ca^{2+} ATPase [PMCA], sarco/endoplasmic reticulum Ca^{2+} ATPase [SERCA]), and various effectors (calmodulin, classic PKC isoenzymes, NFAT and CREB transcription factors; (42)). Members of the Ca^{2+}-signalling toolkit that are functionally expressed in MSCs and differentiating and mature chondrocytes are summarised in Table 1.

As noted above, Ca^{2+} signalling pathways control nearly every aspect of cellular processes, including proliferation and differentiation (41). Consequently, since these are very important functions of MSCs, extensive research concerning the Ca^{2+} homeostasis of these cells has been started at the beginning of the previous decade, including Ca^{2+} entry and elimination processes and signalling pathways that translate Ca^{2+} signals into cellular responses. Undifferentiated MSCs are *per se* non-excitable cells; however, as a result of their differentiation programme, they can give rise to many different cells types, including excitable cells (*e.g.*, muscle and nerve cells; (43)). Despite accumulating knowledge concerning the Ca^{2+} homeostasis and ion channel assemblage (this latter is also referred to as the 'channelome') of non-excitable cells including mature chondrocytes and the growing evidence regarding their involvement in the regulation of metabolic processes and life cycle of these cells, the contribution of these pathways and various ion channels in the differentiation programme is not yet fully understood. Based on that, Ca^{2+} signalling pathways are expected to play pivotal roles in governing the differentiation of non-excitable MSCs to various excitable and non-excitable cells. Moreover, it is also anticipated that a transition in ion channel composition also accompanies (and probably regulates) differentiation of MSCs. As shown in Figures 3 and 4, mesenchymal stem cells and mature chondrocytes possess a distinct, although partially

Table 1. Elements of the global Ca^{2+}-signalling toolkit expressed by mesenchymal stem cells, differentiating and mature chondrocytes. References are shown in brackets

	Mesenchymal Stem Cells	Differentiating Chondrocytes	Mature Chondrocytes
Ca^{2+} Entry From Extracellular Sources			
Voltage-operated Ca^{2+} channels (VOCCs)	L-type Ca^{2+} channels (46, 49,50, 51)	$_{1S}$ subunit in VOCCs (52) $Ca_V1.2$ and 3.2 (53) VOCCs (56)	VOCCs (not specified) (54) VOCCs (not specified) (55)
Ligand-operated Ca^{2+} entry			
Purinergic Receptors	$P2Y_1$ (58) $P2X_6$, $P2Y_4$, and $P2Y_{14}$ (59) P2X receptors (60) (Reviewed by (61))	$P2Y_1$ (62) ($P2X_1$, $P2X_5$, $P2X_7$) and metabotropic ($P2Y_1$, $P2Y_2$, $P2Y_4$) (63)	P2Y receptors (64, 65, 68) P1 and P2 receptors (66) P2X (67)
N-methyl-D-aspartate (NMDA) receptors	(70)	NR1, NR2D and NR3A subunits (74); NR1 (33)	NR1, NR2A and NR2B (75, 76)
Transient Receptor Potential (TRP) receptors	TRPC4 (46); TRPC1 and 2 (48); TRPM7 (79); TRPC1 and 4 (80); TRPC1, 2, 4 and 6 (81)	TRPV4 (82)	TRPC1, 3 and 6; TRPM5 and 7; TRPV1 and 2 (83); TRPV4 (78, 84, 85); TRPV5 (86)
Ca^{2+} Entry From Internal Stores			
Inositol-1,4,5-trisphosphate receptor (IP_3R)	IP_3R type 1, 2 and 3 (46, 48)	PLC–IP_3R pathway (63, 89)	PLC–IP_3R pathway (87)
Ryanodine receptor (RyR)	No RyR expression and function (46, 48)	Weak RyR expression, no RyR function (63, 89)	RyR in chondrocyte death (88)
Store-Operated Ca^{2+}-Entry	I_{SOC} via TRPs (46, 48); STIM1 and Orai1-dependent SOCE (93, 94)	Dependence on extracellular Ca^{2+}; SOCE (89)	Dependence on availability of internal Ca^{2+} stores (91)
Arachidonate-Regulated Ca^{2+}-Sensitive (ARC) Channels	unknown	unknown	unknown
Ca^{2+} Elimination Processes			
Plasma Membrane Ca^{2+}-ATPase (PMCA)	PMCA4 (98)	unknown	unknown
Na^+/Ca^{2+}-exchanger (NCX)	(98)	unknown	(100)
Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)	(46)	(89)	(128)

overlapping set of Ca^{2+} ion channels or receptors involved in the regulation of their Ca^{2+} homeostasis.

7. CA^{2+} ENTRY PROCESSES IN MSCS AND IN DIFFERENTIATING OR MATURE CHONDROCYTES

Ca^{2+} can enter the cytosol mainly from two sources: either from the extracellular space or from intracellular Ca^{2+} stores. The high number of plasma membrane Ca^{2+} channels are conventionally divided into four groups: voltage-operated calcium channels (*e.g.*, L- and T-types); ligand- or receptor-operated calcium channels (*e.g.*, *N*-methyl-D-aspartate or NMDA type glutamate receptors, transient receptor-potential (TRP) channels, ionotropic purinergic P2X Ca^{2+} channels); second messenger-operated channels (*e.g.*, arachidonate-regulated Ca^{2+} channel); and Ca^{2+} release-activated Ca^{2+} channels (CRAC) that couple extracellular Ca^{2+} entry and intracellular Ca^{2+} release via the activation of store-operated Ca^{2+} channels (42). The other main source of cytosolic Ca^{2+} is release from intracellular Ca^{2+} stores, which is regulated by two distinct Ca^{2+} channels: the inositol-1,4,5-trisphosphate receptors (IP_3R) and the ryanodine receptors (RyR; (44)). Ca^{2+} release from internal stores is agonist-dependent and voltage-independent. For the process of store-operated Ca^{2+} entry (SOCE), whereby Ca^{2+} influx from the extracellular space is activated in response to depletion of intracellular Ca^{2+} stores in the ER, interaction between STIM (a Ca^{2+} sensor in the ER) and the Orai family (plasma membrane Ca^{2+} channels) plays an important role (for a recent review, see (45)).

7.1. Voltage-operated Ca^{2+} entry pathways

Ca^{2+} influx pathways across the plasma membrane represent the main entry of Ca^{2+} into the cytosol of most cell types; moreover, release from intracellular Ca^{2+} stores also plays important roles. Since human MSCs (hMSCs) have become commercially available, Ca^{2+} signalling pathways (including Ca^{2+} entry processes) have been thoroughly investigated. In the seminal work of Kawano and co-workers, two main Ca^{2+} entry pathways were reported in undifferentiated hMSCs: voltage-operated Ca^{2+} channels (VOCCs) and store-operated Ca^{2+} channels (SOCs; (46)). As SOCs are responsible for Ca^{2+} entry in majority of non-excitable cells examined, presence of a store-operated Ca^{2+} current (I_{SOC}) in hMSCs that is highly selective for Ca^{2+} is in accordance with previous findings (47).

VOCCs can be subdivided into several subtypes based on their characteristics: L- ($Ca_V1.1$, 1.2, 1.3, 1.4), P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), R- ($Ca_V2.3$) and T-type ($Ca_V3.1$, 3.2, 3.3) channels (44). Evidence of the functional expression of L-type Ca^{2+} channels was reported based on their sensitivity to dihydropyridines (although only 15% of cells measured exhibited small inward currents that could be blocked by nicardipine), but no N-type Ca^{2+} channels could be detected in hMSCs (46). Similar findings were reported in mouse embryonic stem cells (mESCs), where a capacitive Ca^{2+} entry, rather than influx via VOCCs was found to be the main basal Ca^{2+} entry pathway (48). In fact, no mRNA expression of L-, T- or P/Q-type Ca^{2+} channels could be detected in the latter study. In spite of the observations that VOCCs seem to contribute to Ca^{2+} entry to a lesser extent compared to store-operated Ca^{2+} entry processes in hMSCs, L-type Ca^{2+} channels were also identified in human embryonic stem cells (49) and in rat MSCs (50). Moreover, Zahanich and colleagues demonstrated that although L-type Ca^{2+} channels are involved in the maintenance of Ca^{2+} homeostasis in differentiated osteoblasts, functional expression of these

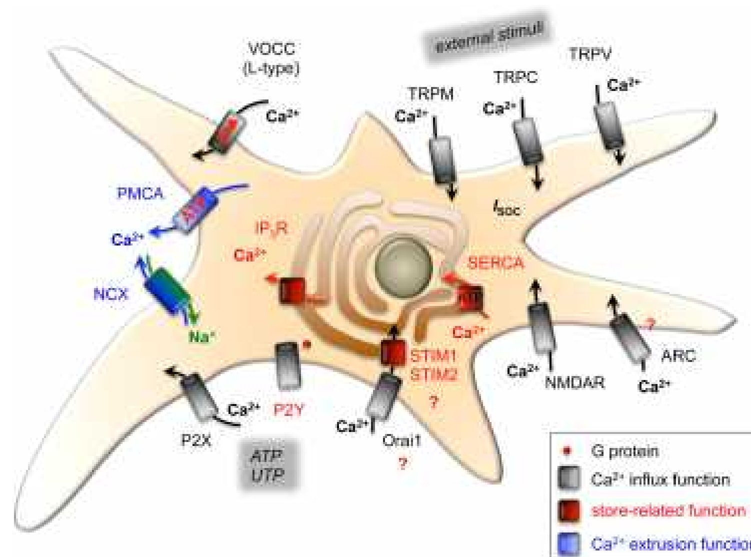

Figure 3. Elements of the global Ca^{2+} signalling toolkit functionally expressed by mesenchymal stem cells. This figure demonstrates the main Ca^{2+} ion channels and receptor proteins identified to date on MSCs. Ion channels or cell surface receptors involved in Ca^{2+} influx or release are shown in black; Ca^{2+} extrusion functions are in blue; and intracellular release and Ca^{2+} pump proteins are in red. Arrows indicate direction of Ca^{2+} ion flow across various channels, pumps and/or exchangers. These data are summarized more fully in Table 1. For references, please see Table 1 or text. Hypothetical Ca^{2+} ion channels and/or receptors are marked by question marks. (ARC: arachidonate-regulated Ca^{2+}-sensitive channel; NCX: Na^{+}/Ca^{2+} exchanger; NMDAR: N-methyl-D-aspartate receptor; P2X: ionotropic purinergic receptor; P2Y: metabotropic (G protein coupled) purinergic receptor; PMCA: plasma membrane Ca^{2+} ATPase; STIM: stromal interaction protein; TRPC, TRPM, TRPV: transient receptor potential canonical, melastatin, vanilloid; VOCC: voltage-operated Ca^{2+} channel).

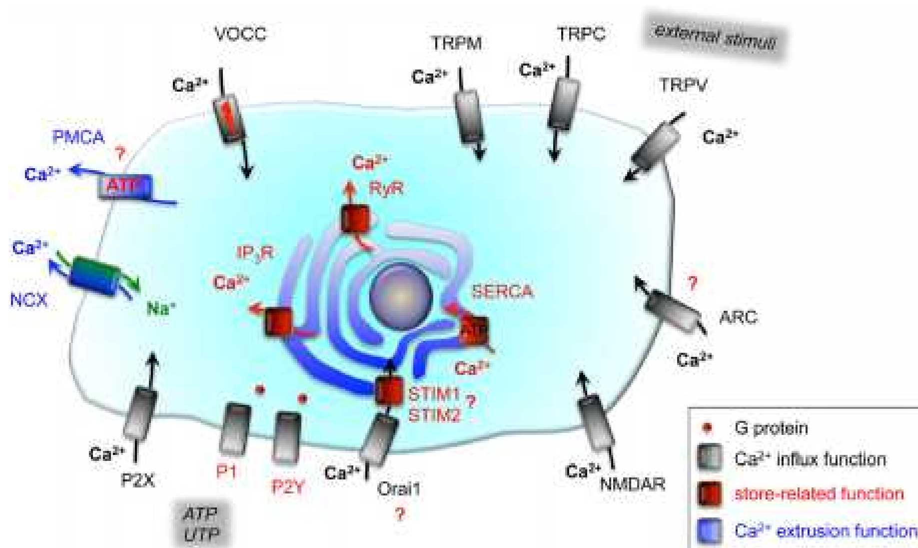

Figure 4. Elements of the global Ca^{2+} signalling toolkit functionally expressed by mature chondrocytes. This figure summarises the main Ca^{2+} ion channels and receptor proteins identified to date on mature chondrocytes. Ion channels or cell surface receptors involved in Ca^{2+} influx or release are shown in black; Ca^{2+} extrusion functions are in blue; and intracellular release and Ca^{2+} pump proteins are in red. Arrows indicate direction of Ca^{2+} ion flow across various channels, pumps and/or exchangers. These data are summarized more fully in Table 1. For references, please see Table 1 or text. Hypothetical Ca^{2+} ion channels and/or receptors are marked by question marks. (ARC: arachidonate-regulated Ca^{2+}-sensitive channel; NCX: Na^{+}/Ca^{2+} exchanger; NMDAR: N-methyl-D-aspartate receptor; NMDAR: N-methyl-D-aspartate receptor; P1: adenosine receptor; P2X: ionotropic purinergic receptor; P2Y: metabotropic (G protein coupled) purinergic receptor; PMCA: plasma membrane Ca^{2+} ATPase; STIM: stromal interaction protein; TRPC, TRPM, TRPV: transient receptor potential canonical, melastatin, vanilloid; VOCC: voltage-operated Ca^{2+} channel)

channels is not required for osteogenic differentiation of hMSCs (51).

Expression of voltage-operated Ca^{2+} channels has also been reported during chondrogenesis. In organoid cultures of limb buds derived from early mouse embryos, VOCCs were detected to co-localise with beta-1 integrin, Na/K-ATPase and epithelial sodium channels in mechanoreceptor complexes in developing chondrocytes (52). Indeed, expressions of various Ca_V1.2 and 3.2 channel subunits were detected in chondrocytes in developing murine embryos by immunohistochemical staining procedures; moreover, protein expressions of the same L-type Ca^{2+} channels were identified in the ATDC5 chondrogenic cell line (53). Voltage-gated Ca^{2+} channels have long been known to be expressed by articular chondrocytes and contribute to elevation of cytosolic Ca^{2+} concentration by insulin-like growth factor-1 (54). Xu and co-workers recently demonstrated that Ca^{2+} influx via VOCCs is also necessary for signalling pathways in stimulated articular chondrocytes (55). Preliminary findings of our laboratory also implicate the involvement of various voltage-gated Ca^{2+} channels during *in vitro* chondrogenesis in chicken HDC established from distal limb buds of 4-day-old chicken embryos. In contrast to murine ESCs, where no rise in cytosolic Ca^{2+} concentration was observed when cells were exposed to external solution containing high concentration of K^+ (48), chondrifying cells in chicken HDC exhibited large Ca^{2+} transients by exposure to high concentrations of K^+ (56), implicating the functional expression of VOCCs. These differences may be the consequence of the more differentiated state of cells in HDC compared to embryonic stem cells. Notwithstanding these interesting results, further studies are required to clarify the precise role of voltage-dependent Ca^{2+} channels during chondrogenesis.

7.2. Ligand-operated Ca^{2+} entry pathways

7.2.1. Purinergic signalling pathways

Besides VOCCs, the other main Ca^{2+} entry pathway across the plasma membrane is via ligand-operated Ca^{2+} channels (44). A typical example of these channels is the family of purinergic receptors. Activated by extracellular nucleotides, purinergic receptors are conventionally divided into two major types: P1 receptor families are sensitive to adenosine, while ligands for P2 receptor families include ATP, ADP, and UTP. P2 receptors are further divided into two major receptor subtypes: P2Y and P2X. Members of the metabotropic P2Y receptor subtype are 7 transmembrane domain-containing G-protein coupled receptors (GPCRs) whose activation leads to the release of intracellular Ca^{2+} from IP_3-sensitive Ca^{2+} stores. The ionotropic P2X receptors are ATP-gated *bona fide* ion channels that allow Ca^{2+} influx across the plasma membrane (57). In hMSCs, Kawano and colleagues found that GPCRs, mainly metabotropic purinergic (P2Y) receptors participated in the regulation of Ca^{2+} homeostasis, by an autocrine-paracrine ATP signalling mechanism (58). ATP was reported to be secreted from hMSCs via hemi-gap junctions to stimulate $P2Y_1$ receptors in the plasma membrane. Moreover, in a more recent study the subtypes $P2X_6$, $P2Y_4$, and $P2Y_{14}$ were found to be pivotal regulators in adipose tissue-derived adult MSC commitment during adipogenic and osteogenic differentiation (59). The functionality of purinergic receptors in human skin-derived MSCs was demonstrated by Orciani and colleagues when they reported that MSCs responded to administration of ATP by elevation of cytosolic concentration via P2X receptors (60). In spite of the growing evidence regarding the emerging role of purinergic signalling in stem cells (for a review, see (61)), as the majority of work was performed on neuronal or muscle precursors, current knowledge is still considerably limited concerning the possible involvement of extracellular nucleotide-gated signalling during the differentiation of MSCs towards other lineages.

The purinergic concept in the regulation of chondrogenesis was first suggested by Meyer and co-workers in 2001 (62). They reported that $P2Y_1$, a GPCR whose activation leads to the release of Ca^{2+} from intracellular stores, was a negative regulator of chondrogenesis in chicken HDC as overexpression of this receptor has led to attenuated cartilage matrix production in these cell cultures. Moreover, they also showed evidence that these cells released ATP, the ligand of $P2Y_1$ into the culture medium. In line with these experiments, results of our laboratory also support the hypothesis that purinergic signalling is involved in the chondrogenic differentiation of chicken mesenchymal cells (63). In contrast to the above findings, we concluded that ionotropic P2X receptors, rather than metabotropic P2Y receptors, were primarily involved in positively regulating *in vitro* chondrogenesis in chicken limb bud-derived HDC, and we confirmed that the plasma membrane expression of $P2X_4$ receptor was at least partially responsible for a major Ca^{2+} influx required for differentiation of chondroprogenitor mesenchymal cells in this system. The other ionotropic ($P2X_1$, $P2X_5$, $P2X_7$) and metabotropic ($P2Y_1$, $P2Y_2$, $P2Y_4$) purinergic receptors whose expression was detected in the plasma membrane are probably involved in the maintenance of basal cytosolic Ca^{2+} concentration (63).

The functional characterisation of purinergic signalling pathways in articular chondrocytes have been performed almost two decades ago (64, 65). Mature chondrocytes have also been reported to express both P1 and P2 purinoreceptor genes (66) and that detailed analyses revealed that members of both P2X (67) and P2Y (68) purinoreceptors were expressed and functional in chondrocytes. Although their role played in Ca^{2+} homeostasis of adult cartilage cells is not fully characterised, their involvement in chondrocyte mechanotransduction pathways is implicated (see below).

7.2.2. *N*-methyl-D-aspartate receptor mediated pathways

L-glutamate is the major excitatory neurotransmitter that can activate ionotropic (iGluR) and metabotropic (mGluR) receptors. Ionotropic glutamate receptors are *bona fide* ligand-gated non-selective cation channels, and based on their selective pharmacological agonists they are subdivided into alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate receptors (NMDAR). While AMPA

and kainate receptors are mostly permeable to K^+ and Na^+, NMDARs are primarily permeable to Ca^{2+} (44). NMDARs are heterotetrameric complexes that are comprised of two obligatory NR1 subunits and two others of four distinct NR2 (NR2A, NR2B, NR2C or NR2D) and/or two NR3 (NR3A or NR3B) subunits (for a recent review, see (69)).

Conventionally, glutamate signalling has been considered as the primary excitatory pathway in the central nervous system; therefore, the involvement of NMDARs in the regulation of differentiation, survival and electrophysiological properties has been extensively investigated in neurons (70). However, glutamate receptor expression and function in non-neuronal tissues is also reported (for a review, see (71)). Interestingly, functional characterisation of glutamaterg signalling pathways, including NMDAR expression and function remains elusive in undifferentiated mesenchymal and embryonic stem cells; these pathways have only been investigated during differentiation into neural precursors and nerve cells (70). Nonetheless, owing to the emerging role of NMDARs in non-excitable cells, the importance of glutamaterg signalling in MSCs, at least partially via NMDARs, can be implicated.

Recent findings demonstrated the importance of glutamaterg signalling in skeletal tissues. NMDARs have proved to play a complex role in bone remodeling (72) and have been reported to be involved in differentiation of osteoblasts and osteoclasts (73). In *ex vivo* organotypic cultures of fetal mouse tibias, Takahata and colleagues demonstrated that mRNAs for the NR1, NR2D and NR3A NMDAR subunits were constitutively expressed in developing chondrocytes and that glycine probably acts on these NMDARs to induce terminal differentiation of these cells (74). Mature human articular chondrocytes have also been reported to express NMDARs (75) and that they are active components of mechanotransduction pathways (76). In accordance with these results, we were also able to confirm the mRNA and protein expressions of the NR1 NMDAR subunit in chicken HDC. Furthermore, NR1 subunit expression was found to be affected by mechanical stimulation in chondrogenic cells (33). Nonetheless, the importance of glutamate signalling in the regulation of cartilage development is yet to be clarified.

7.2.3. Transient Receptor Potential (TRP) pathways

Accumulating evidence suggests that transient receptor potential (TRP) channels play prominent roles in regulating the intracellular Ca^{2+} concentration in non-excitable cells. TRPs are clearly remarkable proteins: they constitute a large and functionally versatile family of cation-conducting channels, and at the same time, they have also been considered as unique cell sensors. Of the more than 50 TRP channels identified thus far in many species, the 28 mammalian TRP channels are conventionally divided into 6 subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), mucolipin (TRPML), polycystin (TRPP), and TRPN (“no mechanoreceptor potential C”); most of them comprises numerous members (77). Members of the TRP subfamilies are reported to be expressed in majority of cells and tissues examined and found to regulate many basic physiological processes, including nociception, temperature and osmosensation or muscle contraction. Functioning as non-selective cation channels, the molecular structure of TRPs highly resembles that of voltage-operated ion channels; upon activation, TRPs can cause cell depolarisation. TRPs may not only act as *bona fide* cation channels that can enable a rise in cytosolic free Ca^{2+} concentration, but (1) their activity may influence gating of VOCCs mainly in excitable cells; (2) TRP channels can regulate the driving forces for Ca^{2+} entry, mainly in non-excitable cells, by de- or hyperpolarizing the plasma membrane via Ca^{2+} -dependent activation of other ion channels; and (3) TRPs themselves are influenced (activated or inhibited) by changes of intracellular Ca^{2+} concentration (78). While the majority of TRPs are located in the plasma membrane and allow for Ca^{2+} influx (all the TRPCs, all TRPVs, TRPM1, 2, 3, 6, 7, and 8, TRPA1, TRPP2, 3, and 5 and TRPML1, 2, and 3), some of the TRP channels are reported to be mainly localised in intracellular membranes (such as TRPML1 and TRPP2) (77).

Kawano and colleagues have detected the mRNA expression of TRPC4 (and not that of TRPC3, 5 and 7) in hMSCs and implicated its involvement in the store-operated Ca^{2+} currents characteristic to these cells (46). As the function of TRP channels has primarily been investigated in the nervous system especially concerning nociception, currently available data regarding the expression and role of various TRPs in mesenchymal stem cells is mostly limited to neurogenesis. Nonetheless, the importance of TRPM7 in the survival of bone marrow-derived MSCs has been recently reported (79). Weick and co-workers demonstrated that TRPC1 and TRPC4 act as molecular targets for controlling neurite elongation in human embryonic stem cell-derived neurons (80). That MSCs express canonical TRPC1, 2, 4 and 6 mRNA and that TRPC1 may be involved in stem cell proliferation has been recently shown (81).

Data available concerning the putative role of TRPs during chondrogenesis is also sparse. Muramatsu and co-workers revealed that TRPV4 is a positive regulator of Sox9: besides the fact that the mRNA expression profile of TRPV4 highly resembled those of chondrogenic marker genes in the chondrogenic cell lines ATDC5 and C3H10T1/2, pharmacological activation of TRPV4 induced Sox9 reporter activity (82). Furthermore, they also showed that the Ca^{2+}–calmodulin pathway was required for this process. As far as TRP channel expression in mature chondrocytes is concerned, Gavenis and colleagues have reported that articular chondrocytes isolated from knee joints of osteoarthritic patients express various TRP ion channels (83). That TRPV4 is an osmotically active ion channel in mature chondrocytes and that it is also an important component of chondrocyte mechanotransduction pathways has been reported (84, 85). Furthermore, resting membrane potential in chondrocytes was found to be at least partially regulated by TRPV5 (86).

Although the exact role of TRPs in chondrocyte differentiation and maturation is still not well understood,

given that TRPV4 is a stretch-activated channel that allows for stretch-activated Ca^{2+} entry (78), it may be hypothesised that TRPV4 ion channels (and possibly other stretch-activated TRPs) may be key regulators of chondrogenesis by translating mechanical forces into Ca^{2+} mobilization.

7.3. Ca^{2+} Release from internal stores and store-operated Ca^{2+} entry

Ca^{2+} release from intracellular Ca^{2+} stores (primarily from the ER) represents the other main source of cytosolic Ca^{2+} besides influx from the extracellular space across the plasma membrane. Investigation of Ca^{2+} release from internal stores in MSCs has led to the conclusion that IP_3Rs are the primary factors that allow for Ca^{2+} release from the ER (although to a variable extent, all three IP_3R subtypes were found to be expressed at the mRNA level in hMSCs), while RyR, the other main Ca^{2+} release channel was not found to be involved in this process as its functional expression (either at the mRNA level or by local application of the RyR-agonist caffeine during Ca^{2+} measurements) could not be proved in hMSCs (46). Murine ESCs have proved to utilise the same Ca^{2+} release pathway: mRNA expressions of IP_3R subtypes 1, 2 and 3 were confirmed, but no mRNAs for ryanodine receptors could be detected in these cells (48). Mature chondrocytes were also reported to possess internal Ca^{2+} stores that are releasable via the activation of the PLC–IP_3R pathway (87). The involvement of RyR in impact-induced chondrocyte death in mature equine chondrocytes was reported by Huser and Davies (88). Our findings are in accordance with the above results in that internal Ca^{2+} stores in developing chondrocytes of chicken HDC also contained IP_3Rs, rather than RyRs, and the PLC–IP_3R pathway was found to be functioning in these cells. Nonetheless, internal Ca^{2+} stores seemed to contain relatively low amounts of releasable Ca^{2+} in differentiating cells of chicken HDC (63, 89).

In electrically non-excitable cells, Ca^{2+} entry pathways are dominated by store-operated Ca^{2+} entry (SOC) channels; by definition, their activation is dependent on the depletion of internal Ca^{2+} stores (90). Given that human MSCs are essentially non-excitable cells, Kawano and co-workers reported that hMSCs primarily utilise SOC pathways (I_{SOC}) rather than influx via VOCCs, although they refrained from characterising these molecules in detail (46). The store-operated Ca^{2+} channels are activated by store depletion, which has been confirmed by local administration of cyclopiazonic acid (CPA) or thapsigargin (SERCA inhibitors) to MSCs during single cell Ca^{2+} measurements. Attempts have been made towards identifying the molecules for I_{SOC}, and the role of TRP family proteins (most importantly, TRPC4) was implicated. The same group reported that I_{SOC} is the main Ca^{2+} entry pathway also in murine ESCs and found that TRPC1 and TRPC2 might be responsible for Ca^{2+} entry in these cells (48). Signs of SOC pathways have been recorded in differentiating chicken chondroprogenitor mesenchymal cells in HDC during single cell Ca^{2+} measurements (89); however, our results contradict observations reported in human and murine stem cells. Cells in chicken HDC were found to primarily depend on the availability of free extracellular Ca^{2+}: chelation of extracellular Ca^{2+} by EGTA almost completely abolished cartilage matrix production by preventing differentiation and proliferation of these cells, and at the same time significantly reduced cytosolic free Ca^{2+} concentration. Also, removal of available extracellular Ca^{2+} during single cell fluorescent Ca^{2+} measurements immediately resulted in slightly lower Ca^{2+} levels (approx. 10 nM), reflecting on the sensitivity and dependence of Ca^{2+} homeostasis of these cells on extracellular Ca^{2+}. Furthermore, long-term treatments with CPA did not have any effect on cartilage matrix production, nor on proliferation rate of cells, also supporting the hypothesis that the contribution of internal Ca^{2+} stores to the Ca^{2+} homeostasis of differentiating chicken chondrocytes is insignificant (89). To the contrary, in mature porcine articular chondrocytes, the importance of internal Ca^{2+} stores in the regulation of Ca^{2+} homeostasis was implicated, without a significant contribution from Ca^{2+} influx (91); these findings also support the concept that a profound change in Ca^{2+} homeostasis, as well as in the Ca^{2+}-signalling toolkit complement may characterise each step of differentiation processes from undifferentiated pluripotent cells to committed progenitors and mature cells.

Although the existence of SOC pathways that represent the major Ca^{2+} entry to non-excitable cells (via Ca^{2+} release-activated Ca^{2+} channels or CRAC) has long been known, the characterisation of the molecular machinery that orchestrates SOCE took almost 20 years to accomplish. Two major SOCE components have been identified; the stromal interaction molecules (STIM) and the Orai ion channel family (for a review, see (92)). STIM1 is a Ca^{2+} sensor protein located in the ER membrane possessing an EF-hand domain that activates SOCE upon Ca^{2+} store depletion, whereas its homologue STIM2 is mainly involved in the maintenance of basal cytosolic and ER Ca^{2+} levels. Upon store depletion, STIM1 redistributes to sub-plasma membrane puncta, where it co-localizes with Orai1 to form the basic subunit required for SOCE. The close apposition of the ER and plasma membrane at the STIM1–Orai1 clusters enables SERCA pumps to rapidly and efficiently refill the ER Ca^{2+} stores (92). In spite of previous results providing evidence that SOCE pathways are responsible for the main Ca^{2+} processes in MSCs, the characterisation of these molecules in undifferentiated mesenchymal stem cells has not been performed yet. However, Darbellay and colleagues reported that STIM1 and Orai1-dependent SOCE is involved in myoblast differentiation in myogenic stem cells (93). In more committed endothelial progenitor cells, mRNA expressions of molecules that mediate SOCE, including TRPC1, TRPC4, Orai1, and STIM1, were identified (94). As yet, no data are available concerning the expression and role of these proteins either in developing or in mature chondrocytes.

Over a decade ago, a novel way of non-capacitive Ca^{2+} entry pathway has been described in many cell types including both electrically excitable and non-excitable cells that is entirely independent of store depletion and is rather dependent on receptor-mediated generation of arachidonic acid (95). While arachidonate-

regulated Ca^{2+}-sensitive (ARC) channels are implicated to be involved in the modulation of the frequency of agonist-induced Ca^{2+} oscillations (see below), SOC channels determine the amplitude of sustained Ca^{2+} signals. Calcineurin was found to be the key downstream target that differentially relays signals from these two Ca^{2+} channels: oscillatory Ca^{2+} signals generated by the activation of ARC channels fail to increase calcineurin activity, while a long-term sustained rise in cytosolic Ca^{2+} concentration is required for calcineurin activation (96). In spite of the growing body of evidence regarding the activation and function of ARC channels, the molecular characterisation of this novel kind of channels has only recently been revealed and proved to be a pentameric structure primarily made up of Orai1 and Orai3 subunits (97). Owing to the fact that these ARC channels have originally been described only a decade ago (95), their characterisation in MSCs has not been performed yet. In fact, based on experiments by using La^{3+}, a blocker of non-selective cation channels, Kawano and colleagues concluded that an unknown, novel Ca^{2+} entry pathway that is irrespective of store depletion was functioning in hMSCs, which contributed to Ca^{2+} oscillations (98) – this channel (among other candidates) could have well been an ARC channel. Although no data are available regarding the presence of ARC channels in chondrocytes, arachidonate metabolism has long been known to regulate *in vitro* chondrogenesis in chicken limb bud-derived HDC (99); therefore, the involvement of these channels during chondrocyte differentiation may be hypothesised.

8. CA^{2+} ELIMINATION PROCESSES IN MSCS, DIFFERENTIATING AND MATURE CHONDROCYTES

In general, basal cytosolic Ca^{2+} concentration is in the range of 100 nM. In order to eliminate the cellular signal and attenuate Ca^{2+} dependent signalling pathways, restoration of the resting cytosolic Ca^{2+} concentration is required. The plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^{+}/Ca^{2+}-exchanger (NCX) are the primary factors that eliminate cytosolic Ca^{2+} ions towards the extracellular space, while the sarco(endo)plasmatic reticulum-Ca^{2+}-ATPase (SERCA) located within the membrane of ER is involved in the accumulation of cytosolic Ca^{2+} in the intracellular stores. The fourth pump mechanism is the mitochondrial uniporter that pumps Ca^{2+} ions into mitochondria (42).

In hMSCs, Kawano and co-workers were the first to describe the functional expression of PMCA and NCX (98). In this work, they also demonstrated that these Ca^{2+} extrusion mechanisms are important contributors to the maintenance of Ca^{2+} homeostasis in these cells by sustaining oscillatory changes in cytosolic Ca^{2+}. The same group has also reported the functional expression of both PMCA and NCX in mouse ESCs (48). Of the PMCA isoforms, PMCA-1 and PMCA-4 mRNA expression was detected in stem cells, while all three NCX isoforms (NCX-1, -2, and-3) were found to be expressed in these models. In comparison with PMCA and NCX, SERCA and the mitochondrial uniporter only play minor roles in Ca^{2+} elimination processes. Nevertheless, numerous data gained by the application of well-known SERCA inhibitors (thapsigargin, CPA) have indirectly provided evidence that SERCA was active and functioning both in MSCs (46) and in developing chondrocytes (89). In mature articular chondrocytes, only the presence and function of NCX has been reported (100).

9. TEMPORAL CHARACTERISTICS OF CA^{2+} DEPENDENT SIGNALS

Besides the wide variety of the Ca^{2+}-signalling toolkit mentioned above, the precise temporal regulation of cytosolic Ca^{2+} concentration also significantly contributes to the versatility of Ca^{2+} dependent signalling pathways. In certain cell types (*e.g*. cardiac muscle cells or neurons), the effectors respond to changes of cytosolic Ca^{2+} concentration within milliseconds, whereas in other cells the same kinds of changes require seconds or even minutes. Cellular proliferation and differentiation are key processes that both require long-term, sometimes sustained changes in cytosolic Ca^{2+} concentration.

9.1. Day-by-day variation of cytosolic Ca^{2+} concentration

The dependence of *in vitro* chondrogenesis on extracellular Ca^{2+} has long been known (101) as high concentrations of this cation promoted chondrogenic differentiation in chicken HDC as early as the condensation or aggregation phase. Extracellular Ca^{2+} was also reported to modulate cell differentiation during skeletogenesis in chicken embryonic calvaria, where low concentrations enabled chondrogenesis (102). Treatment with the L-type channel-specific blockers nifedipine and verapamil during chondrogenesis in mouse limb bud-derived HDC attenuated differentiation, as well as mineralisation (103). Based on the above results, and given that chondrogenesis is regulated by signalling pathways that are Ca^{2+} sensitive; moreover, as cellular differentiation processes are *per se* dependent on Ca^{2+}, the concept of Ca^{2+} dependent regulation of chondrogenesis has emerged.

In chicken HDC established from limb bud-derived mesenchymal cells, we have reported a day-by-day variation in cytosolic Ca^{2+} concentration measured by single cell fluorescent Ca^{2+} imaging (89). Spontaneous final commitment and differentiation of majority of chondroprogenitor mesenchymal cells occurs on culturing day 3 in this chondrogenesis model and a significant amount of hyaline cartilage is formed by day 6 of culturing. Interestingly, a characteristic peak in cytosolic free Ca^{2+} concentration was found on day 3 (approximately 140 nM, as compared to 100 nM prior to or after this day). Furthermore, the occurrence of this peak in cytosolic Ca^{2+} concentration was found to be indispensable to proper differentiation of cells in HDC: chelation of extracellular Ca^{2+} on culturing days 2 or 3 resulted in significantly lower cytosolic Ca^{2+} concentration, and chondrogenesis, as well as cartilage matrix production was almost completely abolished. Moreover, uncontrolled influx of Ca^{2+} via A23187 Ca^{2+} ionophore had dual effects: a 1.25-fold increase in cytosolic Ca^{2+} concentration significantly

promoted chondrocyte differentiation and matrix production, while a 1.5-fold increase was already detrimental and negatively affected *in vitro* chondrogenesis (89). As these interventions were only effective when applied prior to or during day 3; furthermore, as rate of cellular proliferation has proved to be the most sensitive parameter, Ca^{2+} dependent signalling may primarily exert its chondrogenesis-promoting or inhibitory effects via modulation of proliferation. All these findings support the hypothesis that a sustained rise in cytosolic Ca^{2+} concentration (but only to a certain extent) at the time of final commitment of chondrogenic cells is a prerequisite to chondrogenic differentiation in these cultures.

9.2. Ca^{2+} oscillations in mesenchymal stem cells and chondrocytes

Various cell types (importantly, many types of non-excitable cells) were described to exhibit Ca^{2+} oscillations in response to sustained stimuli. During these oscillations, the increase of cytoplasmic Ca^{2+} levels is in the range of 50–600 nM. It is obvious that the mere existence of Ca^{2+} oscillations adds another dimension besides the amplitude of Ca^{2+} signals: the Ca^{2+} signal now has a frequency (104). The exact physiological significance of Ca^{2+} oscillations in non-excitable cells has not been fully characterised yet; however, these periodic changes have been reported in case of almost every cell type examined and implicated in having a role in controlling many processes, such as fertilisation, proliferation, secretion, muscle contraction, etc. A possible role of oscillatory elevation of cytosolic Ca^{2+} concentration may lie in activation of Ca^{2+}-sensitive transcription factors. During Ca^{2+} oscillations, Ca^{2+} concentration can periodically exceed the activation threshold for transcription factors, whereas a small, constant increase in cytosolic Ca^{2+} concentration cannot accomplish this (105). Thus, by appropriately decoding information embedded in the frequency and/or amplitude of oscillatory Ca^{2+} signals, the differential activation of different genes may drive cells towards specific developmental pathways (106). This is particularly important for MSCs, and it can be hypothesised that oscillatory increases of cytosolic Ca^{2+} concentration are involved in more complex functions during differentiation towards various lineages.

By using Fluo-3 imaging techniques, Kawano and co-workers were the first to describe that undifferentiated hMSCs exhibited spontaneous Ca^{2+} oscillations with an average frequency of 1 oscillation/120 sec (46). These oscillations were sustained for a short period even in the absence of free extracellular Ca^{2+}, although with lower amplitudes, which implicates that intracellular Ca^{2+} stores play important roles in the generation of these oscillatory changes besides Ca^{2+} influx via plasma membrane ion channels. Consistent with this hypothesis, administration of the SERCA blockers thapsigargin or CPA completely abolished these oscillations; moreover, the fact that the non-selective IP_3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) also inhibited oscillations further supported the theory that these oscillations were primarily dependent on the internal Ca^{2+} stores (46). Nonetheless, Ca^{2+} influx is also required for long-term sustainment of these oscillations as Ca^{2+} influx is necessary to refill the intracellular Ca^{2+} stores upon depletion. As far as the contribution of Ca^{2+} extrusion pathways to the Ca^{2+} oscillations is concerned, both PMCAs and Na^+/Ca^{2+} exchangers (NCX) were found to be involved in sustaining Ca^{2+} oscillations as pharmacological inhibition of PMCAs and NCXs with carboxyesin or caloxin 2A1 and Ni^{2+} or KBR7943, respectively, completely blocked these periodic Ca^{2+} transients (98). The same group also reported that an autocrine/paracrine purinergic loop via the metabotropic purinergic receptor $P2Y_1$ is a crucial regulator of oscillations in MSCs (58). In hMSCs, ATP was demonstrated to be secreted from cells via hemi-gap junction channels, thereby triggering and maintaining Ca^{2+} oscillations by an autocrine mechanism.

Ca^{2+} oscillations were also identified and partially characterised in cells of chicken limb bud-derived chondrifying mesenchymal cell cultures by our group (56, 89). However, several features of these oscillations were found to be different from those reported by Kawano and co-workers. First, the periodicity of these oscillations was significantly (approx. 10 times) shorter, with approximately 4–5 transients/minute (Figure 5). Second, 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 oscillations in most of the cells examined. Third, the involvement of VOCCs in the generation and sustainment of these Ca^{2+} oscillations in differentiating chondrocytes has been implicated (56).

As described earlier, Ca^{2+} oscillations can trigger the activation of Ca^{2+}-dependent transcription factors depending on the frequency (NFAT, CREB, etc.) that regulate important cellular functions such as proliferation and differentiation (107). Kawano and co-workers demonstrated that Ca^{2+} oscillations were required for nuclear translocation of NFAT, as blockade of Ca^{2+} oscillations by interfering with the ATP autocrine/paracrine signalling pathway prevented this process. Furthermore, when MSCs differentiated to adipocytes, Ca^{2+} oscillations were not detectable any more, and the nuclear signal for NFAT has also decreased (58). Dephosphorylation by calcineurin is a prerequisite to nuclear translocation of NFAT (108). As calcineurin enzyme activity was found to be differentially regulated by elevated or lower cytosolic Ca^{2+} concentration in differentiating chondrocytes (89), it implies that this phosphatase may be one of the crucial factors by which Ca^{2+} oscillations govern cellular proliferation or differentiation processes. Similar to the above-mentioned results, the frequency of Ca^{2+} oscillations in HDC was found to be decreased at a differentiation state-dependent manner and could not be detected in mature HDC, which implies that Ca^{2+} oscillations are probably involved in the activation of signalling pathways that control differentiation in chondrifying HDC (56). Moreover, the master transcription factor of chondrogenesis, Sox9 has been reported to be transported into the nucleus by calmodulin in a cytosolic Ca^{2+} concentration-dependent manner (109).

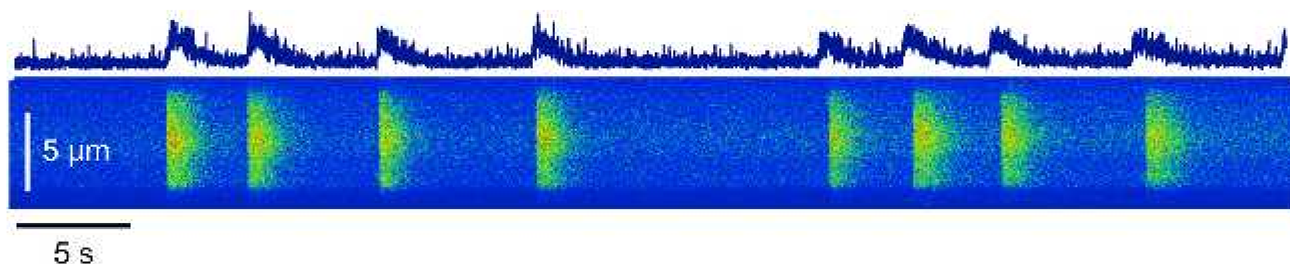

Figure 5. Spontaneous Ca^{2+} events in cells of chondrifying chicken high density micromass cell cultures on culturing day 1. Ca^{2+} transients were determined in Fluo-4-AM loaded cells using Zeiss LSM 510 Meta Laser Confocal Microscope. This representative line-scan record was prepared by using artificial colours, where warmer (towards red) colours represent higher cytosolic Ca^{2+} concentration. The line-scan diagram was recorded at 1.54 ms/line, 512 pixels/line and 8192 lines using a 63× water immersion objective. Fluo-4-loaded cells were excited with a 488 nm argon ion laser and emitted fluorescence was collected at 500—570 nm. Trace above line-scan diagram shows changes in relative fluorescence intensity.

Mature chondrocytes have also been reported to exhibit spontaneous, as well as mechanically induced Ca^{2+} oscillations in sliced pieces of cartilage and these two types of oscillations were found to be different from one another (68). Consistent with data mentioned above regarding the purinergic concept of the regulation of Ca^{2+} oscillations in hMSCs, these oscillatory changes in cytosolic Ca^{2+} concentration in chondrocytes were also mediated by a purinergic signalling pathway. Mature chondrocytes were found to exhibit agonist-induced Ca^{2+} oscillations regulated by mechanisms similar to those in hMSCs; although the rhythmic activity was most importantly triggered by the intracellular stores via activation by ATP, Ca^{2+} influx was required to sustain these oscillations (110).

Although we are far from the detailed characterisation of Ca^{2+} oscillations in non-excitable cells including undifferentiated MSCs, it is clear that different patterns of Ca^{2+} oscillations may differentially regulate signalling pathways that eventually lead to diverse and various responses dependent on the type of the cell and probably on their differentiation stage.

10. CA^{2+} SIGNALLING DURING MECHANOTRANSDUCTION

Mechanical forces have long been known to be key external stimuli that keep musculoskeletal tissues, in particular bone and cartilage in a healthy condition. These tissues are equipped with a not yet fully characterised set of signalling molecules including mechanosensitive ion channels that convert the effects of external stimuli to appropriate biochemical signals during a process called mechanotransduction. The effects of mechanical load have been extensively studied in articular cartilage, in which the unique biomechanical characteristics of the ECM provide a special milieu for chondrocytes that withstand extraordinarily high tensile forces especially in the hip and knee (111). Numerous research groups carried out experiments with bioreactors that were able to apply various loading regimes, including compressive forces (both constant and intermittent), hydrostatic pressure, tensile strain and shear stress (112, 113). Mechanical stimulation has been widely applied on both differentiating and mature musculoskeletal tissues; however, the nature of cellular response to physical stimuli—besides being cell- and tissue-specific—seems to vary according to the type of load. Nevertheless, cyclic compressive loading seems to be the most relevant as it best mimics physiological stimuli on articular cartilage during normal physical activity. That articular cartilage *in vivo* undergoes intermittent compression within the range of 3 to 10 MPa during normal activities has been recently reported (114). Compressive forces have been described to enhance collagen and proteoglycan synthesis in articular chondrocytes; moreover, the involvement of integrin signalling and the MAPK/ERK pathway was also implicated (115). Although both integrins and the ERK pathway are important mediators of chondrocyte mechanotransduction, Ca^{2+} signalling seems to be the major regulator. Various components of the Ca^{2+}-signalling toolkit have been implicated as mediators of mechanotransduction pathways in chondrocytes (Table 2). When exposed to short-term cyclic compression, mature articular chondrocytes responded by the activation of voltage-gated L-type calcium channels and stretch-activated cationic channels, which allowed Ca^{2+} influx resulting in the elevation of cytosolic Ca^{2+} concentration (115). Besides VOCCs, involvement of ligand-gated Ca^{2+} channels in mechanotransduction pathways of chondrocytes has been thoroughly investigated. Of these, Millward-Sadler and co-workers were the first to describe that ATP has a role in the response of adult human chondrocytes to mechanical stimulation, via $P2Y_2$ metabotropic purinergic receptors; moreover, they also found that osteoarthritic cartilage-derived chondrocytes responded differentially to ATP and therefore, the importance of the purinergic pathway in the progression of this disease was implicated (116). More recently, a mechanosensitive purinergic Ca^{2+} signalling pathway via P2Y receptors was also identified in isolated chondrocytes embedded in agarose (117). Moreover, Garcia and Knight provided the first evidence that mechanical loading triggers ATP-mediated purinergic signalling pathways via ATP release through hemichannels in agarose-embedded chondrocytes (118). As outlined above, TRPV4 cation channels are expressed by articular chondrocytes and can be gated by osmotic and mechanical stimuli; indeed, this receptor was found to play a critical role in the maintenance of joint health in a study performed by Clark and co-workers on knee joints of TRPV4-deficient mice (119).

Table 2. Ca^{2+} ion channels or receptors involved in mechanotransduction pathways of differentiating and mature chondrocytes. References are shown in brackets

Ca^{2+} Ion Channel or Receptor Involved in Mechanotransduction	Cell type, reference
VOCCs	Mature chondrocytes (115)
P2Y Purinergic Receptors	Mature chondrocytes (116, 117, 118)
TRPVs (mainly TRPV4)	Mature chondrocytes (119)
NMDARs	Mature chondrocytes (76); differentiating chondrocytes (33)

VOCC: voltage-operated Ca^{2+} channel; TRPV: transient receptor potential vanilloid; NMDAR: *N*-methyl-D-aspartate type glutamatergic receptor

Besides TRPV4, other members of the TRPV ion channel family are also involved in mechanotransduction (for a review, see (120)); however, their role in chondrocytes has not been thoroughly investigated yet. As far as the involvement of NMDA receptors is concerned, role of this ion channel in chondrocyte mechanotransduction pathways has also been implicated (76). Results of our research group also suggest that cyclic compressive force applied during *in vitro* chondrogenesis of chicken HDC influences Ca^{2+} homeostasis via NMDA receptors (33).

While the above results were gained on chondrocytes cultured *in vitro*, data concerning the effects of mechanical stimuli *in vivo* are sparse. Nonetheless, mechanically induced calcium signalling has been confirmed in articular chondrocytes *in situ* (121); moreover, the requirement of a well-defined movement pattern during *in vivo* embryonic joint and articular cartilage formation has also been published (122, 123).

Our current understanding is even more limited regarding mechanotransduction in MSCs. Proper mechanical loading does not only improve the characteristics of developing or mature cartilage but it is implicated to be involved in determining the fate of differentiating mesenchymal cells (124, 125). Mechanical stimulation induces MAPK activation and controls MSC proliferation (126). Application of various mechanical stimuli including cyclic mechanical stress, shear stress or low intensity ultrasound to manipulate MSC differentiation towards different lineages have been recently reviewed by Titushkin and colleagues (127). They envision that mechanical factors are key regulators of MSC differentiation, and that a delicate interplay and tight coupling between Ca^{2+} homeostasis, MAPK pathways and biomechanical remodelling is required for lineage-specific commitment and differentiation of MSCs.

11. CONCLUSIONS

Mesenchymal stem cells represent a new and exciting horizon for cell-based tissue regeneration techniques. To achieve this, recent attempts have been focused on the manipulation of stem cell differentiation towards specific lineages, including chondrocytes. To this end, a better understanding of the molecular machinery governing lineage-specific differentiation is required. Ca^{2+} is generally accepted as a critical player in cellular differentiation processes. As outlined in this review, MSCs are characterised by the functional expression of numerous members of the Ca^{2+}-signalling toolkit. Non-excitable MSCs possess the capability of differentiating to various lineages, including both excitable and non-excitable cells. There is evidence that an adaptation of Ca^{2+}-signalling toolkit to function of mature cells derived from ESCs and MSCs is indispensable. This also supports the concept that a profound change in Ca^{2+} homeostasis, as well as in the assemblage of Ca^{2+}-signalling toolkit characterises differentiation from undifferentiated pluripotent cells to committed progenitors and mature cells. Key targets of general or local changes in cytosolic Ca^{2+} concentration are the extraordinarily wide range of Ca^{2+}-sensitive signalling pathways including—among others—various protein kinase/phosphoprotein phosphatase systems, that may ultimately enable distinct gene expression profiles via differential activation of key transcription factors (NFAT, CREB, etc.), giving rise to lineage-specific differentiation. Therefore, a better understanding of these pathways may ultimately allow us to control MSC differentiation to required cell types. Although appropriate methodologies are readily available to characterise changes in the calcium dynamics and associated signalling pathways, we still do not fully understand whether the observed (and yet-to-be discovered) changes are simply consequences or rather critical prerequisites to MSC differentiation. An in-depth understanding of these mechanisms will undoubtedly lead to a more effective development of MSC-based regenerative tissue engineering techniques for prevention and treatment of articular cartilage disorders and aid strategies for the optimization of such approaches for cartilage repair.

12. ACKNOWLEDGEMENTS

This work was supported by grants from the Hungarian Science Research Fund (OTKA CNK80709) and TÁMOP-4.2.1/B-09/KONV-2010-0007 project implemented through the New Hungary Development Plan, co-financed by the European Social Fund. C.M. is supported by a Mecenatura grant (DEOEC Mec-9/2011) from the Medical and Health Science Centre, University of Debrecen, Hungary. The authors acknowledge Prof. Pál Gergely, Prof. László Csernoch and Prof. László Kovács for their valuable help and comments in writing the manuscript.

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Abbreviations: 2-APB: 2-aminoethoxydiphenyl borate; ACI: autologous chondrocyte implantation; ADP: adenosine diphosphate; AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ARC: arachidonate-

regulated Ca^{2+} channel; ATP: adenosine triphosphate; BMP: bone morphogenic protein; cAMP: cyclic adenosine monophosphate; CD-RAP: cartilage-derived retinoic acid-sensitive protein; CPA: cyclopiazonic acid; CRAC: Ca^{2+} release-activated Ca^{2+} channel; CREB: cAMP responsive element binding protein; ECM: extracellular matrix; ER: endoplasmic reticulum; ERK: extracellular signal-regulated kinase; ESC: embryonic stem cell; FGF: fibroblast growth factor; GAG: glycosaminoglycan; GPCR: G-protein coupled receptor; HDC: high density cell culture; iGluR: ionotropic glutatame receptor; IP_3R: inositol-1,4,5-trisphosphate receptor; JNK: jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; mGluR: metabotropic glutatame receptor; MSC: mesenchymal stem cell; N-CAM: neural cell adhesion molecule; NCX: Na^+/Ca^{2+}-exchanger; NFAT: nuclear factor of activated T-lymphocytes; NMDAR: *N*-methyl-D-aspartate type glutamate receptors; OA: osteoarthritis; PG: proteoglycan; PKA: protein kinase A; PKC: protein kinase C; PMCA: plasma membrane Ca^{2+} ATPase; PP1, PP2A and PP2B: phosphoprotein phosphatase 1, 2A and 2B; RyR: ryanodine receptor; SERCA: sarco/endoplasmic reticulum Ca^{2+} ATPase; SOCE: store-operated Ca^{2+} entry; STIM: stromal interaction molecule; TGF-beta: transforming growth factor-beta; TRP: transient receptor-potential channel; TRPA: ankyrin TRP; TRPC: canonical TRP; TRPM: melastatin TRP; TRPM: mucolipin TRP; TRPN: “no mechanoreceptor potential C” TRP; TRPP: polycystin TRP; TRPV: vanilloid TRP; UTP: uridine triphosphate; VOCC: voltage-operated Ca^{2+} channel

Key Words: Mesenchymal Stem Cells, Chondrocytes, High Density Cultures, Ca^{2+} toolkit, Oscillations, Ion Channels, Review

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