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Current knowledge of Pituitary adenylate cyclase activating polypeptide (PACAP) in articular cartilage

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Summary:

Pituitary adenylate cyclase activating polypeptide (PACAP) is an evolutionally well conserved neuropeptide, mainly expressed by neuronal and peripheral cells. It proves to be an interesting object of study both for its trophic functions during the development of several tissues and for its protective effects against oxidative stress, hypoxia, inflammation and apoptosis in different degenerative diseases. This brief review summarises the recent findings concerning the role of PACAP in the articular cartilage. PACAP and its receptors are expressed during chondrogenesis and are shown to activate the pathways involved in regulating cartilage development. Moreover, this neuropeptide proves to be chondroprotective against those stressors that determine cartilage degeneration and contribute to the onset of osteoarthritis (OA), the most common form of degenerative joint disease. Indeed, the degenerated cartilage exhibits low levels of PACAP, suggesting that its endogenous levels in adult cartilage may play an essential role in maintaining

physiological properties. Thanks to its peculiar characteristics, exogenous administration of PACAP could be suggested as a potential tool to slow down the progression of OA and for cartilage regeneration approaches.

Keywords: PACAP, articular cartilage, chondrogenesis, osteoarthritis, cartilage regeneration.

Introduction

PACAP is a neuropeptide, belonging to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon peptide superfamily, isolated for the first time in 1989 from ovine hypothalamus extracts and further recognized as an activator of adenylate cyclase in rat anterior pituitary cell cultures (Miyata et al., 1989). PACAP appears to be highly and evolutionarily conserved, indeed its structure has been examined in many animal species (Vaudry et al., 2009). PACAP has two biologically active isoforms: PACAP-38 and PACAP-27, respectively composed of 38 and 27 amino acid residues, (Vaudry et al., 2009), which originate from the same protein precursor, pro-PACAP, and sharing the same N-terminal region. PACAP-38 is the most represented isoform, constituting about 90% of the naturally occurring protein (Miyata et al., 1990). Numerous studies have shown that PACAP carries out several functions in the central nervous system (CNS) (Zhou et al., 2002; Maduna and Lelievre, 2016). For example, during the neurodevelopment process, PACAP regulates proliferation, migration, differentiation and survival of neural cells (Gonzalez et al., 1997; Cameron et al., 2007). Moreover, PACAP shows trophic and protective effects in various neurological disorders, such as Parkinson's disease (Yang et al., 2015), ischemia (Reglodi et al., 2018d), traumatic injury (Toth et al., 2020) and Alzheimer's disease (Yang et al., 2015). In the last years, several studies have focused their attention on the role of this molecule on peripheral organs and other systems, such as gonads (Nakamura et al., 2014), eye (Maugeri et al., 2018a and 2019a), intestine (Pirone et al., 2011; Reglodi et al., 2018c), respiratory system (Hauser-Kronberger et al., 1996), urinary tract (Reglodi et al., 2012), lymphoid organs (Gaytan et al., 1994) and bone (Józsa et al., 2018), as well as in body extracellular fluids (Brubel et al., 2011; Csanaky et al., 2013). All this evidence suggests that PACAP is a multifunctional neuropeptide that regulates different biological processes. In particular, it has been established that the pivotal role of PACAP is to prevent the negative effects of oxidative stress (Douiri et al., 2016), hypoxia (Maugeri et al., 2018b), inflammation (Delgado et al., 2003) and apoptosis (Reglodi et al., 2018a; Maugeri et al., 2019b). PACAP is a ligand of three G-protein coupled receptors: pituitary adenylate cyclase-

activating polypeptide type I receptor (PAC1), vasoactive intestinal polypeptide receptor 1 (VPAC1) and receptor 2 (VPAC2). PACAP shows an affinity for PAC1 100 times greater than VPAC1 and VPAC2 (Jolivel et al., 2009; Vaudry et al., 2009). The binding between PACAP to one of its receptors activates the adenylate cyclase that induces an increase of intracellular cyclic adenosine monophosphate (cAMP) levels, with the consequent of protein kinase A (PKA). The latter can phosphorylate many transcription factors, including cAMP response element-binding protein (CREB), SRY-box transcription factor 9 (SOX9), and RUNX family transcription factor 2 (Runx2) which regulates the expression of numerous genes, involved in a plethora of biological processes (Juhász et al., 2015a). Furthermore, PACAP and its receptors take part in several signal transduction mechanisms. Indeed, they may crosstalk with other signalling pathways such as mitogen-activated protein kinase (MAPK), wingless int1 (WNT), transforming growth factor beta (TGFβ), hedgehog (HH), bone morphogenetic protein (BMP) and Ca2+ dependent pathways (Juhász et al., 2015a; Juhász et al., 2015b). Since PACAP plays an essential role in the development of different tissues (Tsuchida et al., 2014) and in regeneration processes including osteogenesis, chondrogenesis (Juhász et al., 2015a) and spermatogenesis (Reglodi et al., 2018b), its possible use for degenerative disease treatment appears to be an interesting object of study. PACAP also plays an essential role in bone tissue, which is closely associated with articular cartilage and constitutes an equally important element of the joint. In *in vitro* osteogenesis, it has been observed that the administration of PACAP increases the expression of some factors such as BMP, SMADs and Runx2 which are actively involved in this process and augments the calcification and the production of new matrix (Juhász et al., 2014c; Juhász et al., 2016). In addition, the role of endogenous PACAP in *in vivo* bone formation has also been demonstrated. Indeed, in PACAP knockout (KO) mice, the lack of neuropeptide leads to molecular and biomechanical abnormalities of bone matrix, followed by increased bone fragility (Józsa et al., 2018). In this review, the main focus will be given to the recent findings concerning the role of PACAP in articular cartilage tissue, from its function in chondrogenesis to its positive effects in preserving cartilage health during degenerative processes such as OA.

Articular cartilage morphology and triggering factors leading to its degeneration

The articular cartilage is a highly specialized connective tissue that covers the bone ends of diarthrodial joints. Its main function is to provide a continuous, lubricated and friction-free surface for the articulations and to facilitate the transmission of loads to the underlying subchondral bone. It

is mainly composed by peculiar cells called chondrocytes, embedded in a dense extracellular matrix (ECM). These cells are specialized in maintaining the health of the cartilage which is an avascular, alymphatic and aneural tissue. Although, in fact, the consistency of their ECM limits the movements and communication between the cells, the chondrocytes are able to respond to a wide range of external stimuli, such as growth factors, hydrostatic pressure and mechanical forces (Sophia Fox et al., 2009). Chondrocytes constitute about 2% of the total volume of articular cartilage and originate from mesenchymal stem cells (MSCs) through the process of chondrogenesis during development (Carballo et al., 2017), while in adult cartilage chondrocytes mainly originate from sequential mitosis. However, also in adult cartilage, the present MSCs have the capacity to differentiate into mature chondrocytes and to contribute to the local repair of micro-lesions (Alsalameh et al., 2004). The mature chondrocytes play an essential role in development, maintenance and repair of ECM (Richardson et al., 2016; Szychlinska et al., 2019a). The ECM is mainly composed of collagen fibres (60% of dry weight), proteoglycans (5–10%), glycoproteins in smaller quantities and water (Carballo et al., 2017). The most abundant collagen fibre is type II collagen, in fact it represents about 80% of the total collagen in ECM and it is responsible for the tensile strength of the tissue. The remaining collagen isoforms include type IX and XI collagens (15%) and 5% of other collagen types (types III, XII, VI, etc.) (Aigner and Stöve, 2003). The proteoglycans present in ECM are aggrecans, decorin, fibronectin, lumican, lubricin and biglycan. In particular, aggrecans are essential in providing the osmotic resistance which is essential to maintain its unique viscoelastic and mechanical properties (Sophia Fox et al., 2009). Besides the main ECM molecules, chondrocytes also synthetize factors involved in ECM degradation such as metalloproteinase (MMPs), hyaluronidases and aggrecanases, which are essential to preserve the integrity of the ECM, by maintaining the homeostasis between synthesis and degradation mechanisms in physiological conditions (Demoor et al., 2014).

Articular cartilage can be morphologically distinct in four zones (Figure 1): superficial zone, intermediate or middle zone, radial zone or deep zone and calcified zone (Szychlinska et al., 2019b). The superficial zone is characterized by high number of flattened chondrocytes, and type II and IX collagen fibres oriented in parallel to the surface, protecting the layers below from biomechanical stress. It is mainly responsible for the viscoelastic ability to resist tensile tangential stresses generated by the flux of the synovial fluid on the surface during rotational movement of the joint. The intermediate zone represents the largest zone of cartilage and consists of randomly oriented collagen fibres with a limited number of spherical chondrocytes (Newman, 1998). It contains more proteoglycans, which maintain the fluids within the tissue and are responsible for the articular cartilage distinctive compression resistance property, due to the presence of repulsive

forces between electrical negative charges opposing perpendicular compressive strain (Sophia Fox et al., 2009). In the radial zone, the collagen fibres are aligned perpendicular to the surface, while the chondrocytes are organized in columns oriented parallel to them, improving resistance to compressive forces. Finally, the calcified zone connects the cartilage to the bone, by implanting the collagen fibrils of the deep zone to the subchondral bone. In this zone, there is a limited number of hypertrophic chondrocytes (Poole et al., 2001; Johnstone et al., 2013) (Fig. 1). Due to limited cellularity and the lack of nerves, blood and lymphatic vessels, cartilage shows limited capabilities for intrinsic healing and repair. Therefore, the maintenance and health of this tissue is essential for joint function, since it is continuously subjected to severe biomechanical load. It is important to consider that chondrocytes can repair only minor damage of the ECM. Indeed, in proximity to a micro-lesion, the adjacent chondrocytes start to enhance proliferative activity and the formation of cluster of cells, in order to compensate the damage. As a consequence, an increased synthesis of ECM components (proteoglycans and type II collagen) is observed (Redman et al., 2005). However, if the stress conditions or damaging factors exceed the reparative capability of the chondrocytes, the damage could become permanent, leading to degenerative and pathological processes, such as OA. The risk factors leading to cartilage degeneration are multiple and may include aging, oxidative stress, inflammatory processes and excessive or insufficient mechanical stress. Aging is a natural and progressive process characterised by a constant and inescapable decrease of physiological efficiency, along with significant biological changes involving all organs and systems over time (Mobasheri et al., 2015). The aging process in the articular cartilage influences mainly the chondrocytes. It has been observed that the reduction of chondrocyte number is also due to the increase of apoptotic and autophagic processes (Loreto et al., 2009; Rahmati et al., 2017). Moreover, the senescent chondrocytes show hypertrophy and a reduced regenerative capacity (Musumeci et al, 2015; Szychlinska et al., 2017). Besides aging, several molecular pathways involved in the homeostasis of ECM could incur in dysregulation, leading to a disruption of its architecture and a progressive loss of its biomechanical properties (Mobasheri et al., 2015; Rahmati et al., 2017). Another triggering factor is represented by oxidative stress, which can be also age-related, contributing to the health of cartilage (Lepetsos and Papavassiliou, 2016). In pathological conditions, the reactive oxygen species (ROS) are overproduced and act like second messengers contributing to reduced chondrocyte viability (Loeser, 2010). In fact, it has been widely demonstrated that high intracellular levels of ROS generate DNA damage, telomere shortening and mitochondrial dysfunctions, leading to senescence and apoptosis of chondrocytes and MSCs (Toh et al., 2016; Szychlinska et al., 2017). In addition, ROS are responsible for ECM degradation, activating the proteases (Fermor et al., 2007). Several studies have highlighted that ROS, as well as

other stressors, may trigger the inflammatory mechanisms (Lieberthal et al., 2015). A large number of cytokines have been reported to be dysregulated within the damaged joint microenvironment, such as interleukin-1β (IL-1β), IL-6, IL-15, IL-17, IL-18, tumor necrosis factor α (TNF-α) and chitinases (Szychlinska et al., 2016; Di Rosa et al., 2019). They are also responsible for aging and apoptosis of chondrocytes and the ECM breakdown, increasing expression of MMPs, collagenases, aggrecanases and other proteases (Mehanaet al., 2019). Furthermore, it has been demonstrated that the pro-inflammatory cytokines inhibit ECM synthesis by increasing the production of nitric oxide (NO) within the chondrocytes (Mobasheri et al., 2015). Finally, it is also known that altered mechanical loading may negatively contribute to joint cartilage health, leading to structural and functional deficits (Karimi et al., 2015). Atypical mechanical forces are able to stimulate chondrocytes to produce increased levels of inflammatory cytokine, ROS and NO (Rai et al., 2017). The latter induces the apoptotic program in chondrocytes and MSCs, resulting in ECM disruption and impaired regenerative properties of the tissue (Kang et al., 2010) (Fig.1).

PACAP involvement in articular cartilage homeostasis

In the context of altered biological processes within the cartilage, numerous studies evaluated the role of different molecules, whose presence or absence could provide insights into degenerative mechanisms correlated with OA onset (Ravalli et al., 2020; Di Rosa et al., 2014). In addition, studies aimed at evaluating the levels of well-known chondroprotective molecules such as lubricin (Nemirov et al.,2020) or hyaluronic acid (HA) (Fonsi et al., 2019), open new perspectives for a possible strategical therapy based on the mechanisms used by these molecules. In this context, PACAP assumes particular interest as an object of study for its potential protective and regenerative effects on articular cartilage. The presence of PACAP in the cartilage was demonstrated for the first time in cartilaginous canals of femoral head and patella samples from pigs, through immunocytochemistry assay (Strange-Vognsen et al., 1997). Successively, PACAP-immunoreactivity has been detected in other cartilaginous structures, such as epiglottis and laryngeal parts of the pharynx in rats (Kano et al., 2011).

The role of PACAP in the cartilage development

The involvement of PACAP in chondrogenesis was investigated for the first time by Juhasz et al. (Juhász et al., 2014a), using chondrogenic cells in micromass cell cultures, isolated from distal part of limb buds, derived from Ross hybrid chicken embryos of Hamburger–Hamilton stages 22-24.

They showed the mRNAs expression of prepro-PACAP and PACAP receptors, as well as protein expression of PACAP1 in chondrogenic cells. After administration of exogenous PACAP in culture medium, the cell cultures showed higher proliferative activity in association with increased ECM production. The latter was confirmed by increased levels of collagen type II and aggrecans. These observations suggest a possible involvement of the PACAP signalling mechanism during the *in vitro* cartilage developing. This hypothesis was confirmed by increased levels and activity of some factors belonging to the PACAP/PAC1 pathway such as PKA, CREB and SOX9, found after PACAP administration. In fact, it has been demonstrated that PKA/CREB–SOX9 signalling plays an essential role during chondrogenesis (Piera-Velazquez et al., 2007; Juhász et al., 2014b), where SOX9 is a key regulator, which enhances the gene expression of some matrix components such as collagen type 2 and aggrecan core protein (ACAN) (Lefebvre, 2019). In addition, decreased expression and activity of PKA/CREB–SOX9 signaling have been observed in articular cartilage from PACAP KO mice, followed by dysregulation of matrix composition (Szegeczki et al., 2019), confirming the relevance of this pathway in cartilage health. It was also observed that in chicken chondrogenic cells exogenous PACAP leads to increased intracellular Ca^{2+} concentration, which promotes the activation of Serine/threonine-protein phosphatase 2B (PP2B) (Juhász et al., 2014a), another positive regulator of chondrogenesis (Matta et al., 2014). Indeed, PP2B activates the transcription factor of activated T cells 4 (NFAT4), which induces the expression of BMP, (Tomita et al., 2002) consequently activating its signaling pathway, this latter also involved in the differentiation and cell aggregation during the chondrogenesis process (Yoon and Lyons ,2004) (Fig.2). Subsequently, the ability of PACAP to exert chondroprotective effects against oxidative stress was also demonstrated (Juhász et al., 2014a). In fact, the administration of PACAP in the chicken micromass cell cultures under oxidative stress, was able to restore the matrix synthesis and cell proliferation. These data confirm previous studies showing the protective effects of PACAP against oxidative stress during cartilage formation (Douiriet al., 2016; Horvath et al, 2019). The PACAP pathway is also activated by mechanical stimuli during chondrogenesis (Figure 2). In fact, increased levels of preproPACAP and protein levels of PAC1 have been found in chicken chondrogenic cells subjected to mechanical stimuli, represented by uniaxial cyclic compressive forces (Juhász et al., 2015b). In addition, under this type of mechanical stimulus the cells showed a hypertrophic phenotype, mainly regulated by the hedgehog (HH) pathway. The exogenous administration of PACAP was able to limit this phenomenon by modulating the effects of HH signaling. Lower levels of some factors belonging to HH pathways such as Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and GLI family zinc finger 1 (Gli1) transcription factor were observed, whereas an increase of Gli2 and Gli3 levels, acting as repressors of HH signalling was found

(Juhász et al., 2015b). How PACAP takes part in this process is not completely clear, however, it has been shown that the PKA-CREB-SOX9 signaling axis is activated during *in vitro* chondrogenesis by this specific mechanical stimulus (Juhász et al., 2014b). Furthermore, it is widely demonstrated that PKA negatively regulates HH signaling. Indeed, in the absence of HH stimulation, PKA directly phosphorylates the Gli proteins, which act as repressors, blocking the expression of the HH target genes (Niewiadomski et al., 2013). These observations suggest a possible way of interacting between PACAP / PAC1 pathway and HH signaling. PACAP also showed the capability of positively regulating the synthesis of ECM and preserving its integrity during stress conditions like mechanical and oxidative stress, also regulating protease activity (Szentléleky et al., 2019). Indeed, the administration of exogenous PACAP in chondrifying cell cultures exposed to oxidative and mechanical stress together, led to a decrease of expression levels and activity of the main proteases involved in matrix degradation, such as Hyal2 and Hyal4, aggrecanase ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS4) and MMPs, in particular MMP1, MMP7, MMP8 and MMP13, during harmful conditions (Szentléleky et al., 2019). These findings indicate that PACAP is a molecule with beneficial effects for normal cartilage development. Moreover, it is able to protect it from impairment, determined by those stressors that characterise joint disorder developments such as OA.

Chondroprotective effects of PACAP in OA onset

OA is the most common degenerative disease afflicting articular cartilage and leads to pain, stiffness, swelling and loss of joint mobility. OA is a heterogeneous and multifactorial disorder regulated by several complex biological interactions at multiple levels (Ratneswaran et al., 2020). The risk factors leading to OA onset are multiple and may include aging, oxidative stress, inflammatory process and mechanical stress (as previously discussed), as well as gender, genetics, ethnicity and obesity. Due to protective properties and beneficial effects on the *in vitro* cartilage development, previously observed, PACAP may have a fundamental role in protecting adult cartilage from the various insults to which it is subjected and in maintaining its health. To date, there is little knowledge regarding the potential functions of PACAP in preventing the onset of OA. However lower PACAP levels were found in degenerated cartilage from *in vivo* OA rat models (Giunta et al., 2015). Additionally, studies on PACAP knockout (KO) mice highlighted that the lack of PACAP caused an augmented articular cartilage aging with OA-like ECM formation (Szegeczki et al., 2019). Specifically, increased thickness of the calcified hypertrophic zone in association with increased levels of collagen type I and type X, which are especially represented in aging cartilage

and calcified zones, was observed in PACAP KO mice (Szegeczki et al., 2019). Therefore, based on these findings and on the evidence reporting PACAP as the powerful neuropeptide with anti-aging effects (Reglodi et al, 2018a), it is easy to assume that in physiological conditions the endogenous levels of PACAP in adult cartilage may play an essential role in maintaining anti-aging and anti-degenerative properties. Consequently, in pathological conditions an imbalance of PACAP levels may influence the progression of cartilage degeneration. The effects of PACAP on apoptosis and inflammation induced through exposure to increasing concentrations of IL-1β, whose presence appears to be predominant for cartilage degeneration (Wojdasiewicz et al., 2014), were analysed in chondrocyte cultures isolated from healthy articular rat cartilage (Giunta et al., 2015). The presence of exogenous PACAP in chondrocyte culture medium inhibited IL-1β expression and IL-1β-induced apoptosis, decreasing the protein levels of apoptosis intermediaries, such as cleaved caspase-3, BCL2 associated X (BAX) and mediators of inflammation, i-NOS and cytochrome c oxidase II (COX-2) (Giunta et al., 2015). Overall, these results further suggest the potential chondroprotective and anti-apoptotic properties of PACAP during experimentally induced inflammation *in vitro*, suggesting the potential application of PACAP as an agent for the treatment of cartilage degeneration diseases like OA (Figure 3).

PACAP presence in the synovial fluid

The articular surfaces of the bones that constitute diarthrodial joints are enclosed within a joint cavity, which is surrounded by an articular capsule, consisting of an external fibrous membrane and an internal synovial membrane. It is a thin cellular layer composed of synoviocytes, whichlines the joint cavity, acting as a semipermeable filter to regulate the transfer of molecules in and out of the joint. It also produces the synovial fluid (SF), normally present in very small quantities in the synovial cavity. The SF is mainly composed of ultra-filtrate of plasma that contains electrolytes, glucose, immunoglobulins and proteins of hematic origin. In addition, several molecules are produced and released in the SF by synoviocytes (Castrogiovanni et al., 2019; Di Rosa et al., 2019). Among these molecules, HA and lubricin are responsible for the visco-elastic properties of this fluid, while proteinases, collagenases, growth factors and cytokines play the leading role in maintaining cartilage homeostasis in physiological and pathological conditions (Blewis et al., 2007). SF is both a lubricant, aiding joint surface movement, and a source of nourishment for the avascular cartilage layers. Indeed, nutrients and other molecules are transported by the SF, by diffusion generated by joint motion, from the synovial cavity to the chondrocytes, (Tamer, 2013). Recently, molecules secreted in biological fluids, for instance SF, have received greater attention

from research, for their use as potential biomarkers for a better understanding of OA progression (Ravalli et al., 2020). In this context, PACAP could be supposed to be secreted in the SF to perform its chondroprotective functions in the cartilage. Furthermore, the reduction of PACAP may contribute to the progression of cartilage degeneration. Indeed, the decreased levels of this neuropeptide have also been observed in synovial fluid (SF) of OA rats (Giunta et al., 2015). Besides, the presence of PACAP has been encountered in SF of human patients under physio-pathological conditions by Sun et al. (Sun et al., 2019a). The latter observed, for the first time, decreased PACAP levels in SF from patients with primary knee OA when compared to the controls, by using enzyme-linked immunosorbent assay (ELISA). In addition, PACAP concentrations were inversely correlated with MMP-3 and IL-1β levels, while higher PACAP levels were detected after HA administration as a treatment in OA patients, suggesting a possible role of PACAP in OA management. Moreover, lower PACAP levels have also been found in SF from 72 post-traumatic knee OA (PTKOA) patients with anterior cruciate ligament (ACL) injury, when compared with controls (Sun et al., 2019b). Furthermore, in these samples, PACAP levels were inversely correlated with levels of IL-1β and TNF-α, suggesting again a potential PACAP involvement in inflammatory mechanisms following degeneration processes (Sun et al., 2019b) (Figure 3).

Conclusion

To date, the articular cartilage repairing approaches, due to its poor regenerative properties, remain a major challenge both in the clinic and for scientific research. Changes in biomechanical and metabolic features of cartilage, related to several risk factors to which it is subjected, lead to ECM degradation and loss of cellular capability, resulting in progressive tissue degeneration and causing severe disability of the joint. An insufficient tissue repair capacity, followed by a progressive loss of cartilage and remodelling of the underlying subchondral bone, leads to OA onset. The current therapeutic approaches, which help in repairing cartilage lesions and to relieve the pain of the affected joints, are not entirely effective. It is also noteworthy that modern regenerative therapies, such as MSCs-based and Scaffold-based therapies, still present limitations for clinical application, mainly due to the phenotypic instability of the cells and to the poor capacity of the constructs to integrate within the tissue and replicate the characteristics of the native cartilage. On the other hand, thanks to recent and innovative tissue engineering methods, based on a multi-disciplinary approach, cartilage regeneration can be improved by combining the use of MSCs, scaffolds, growth factors, mechanical stimuli, exogenous factors and bioactive molecules. In this context, PACAP, thanks to

its ability to promote chondrogenesis *in vitro,* could have an interesting effective application and might have a beneficial impact on future approaches for cartilage regeneration. Indeed, as previously observed, the administration of exogenous PACAP in cell cultures has been able to increase chondrocyte proliferation and differentiation, to stimulate the synthesis of new ECM components and to maintain its homeostasis (Table 1). For these reasons, it may be used as an exogenous agent to promote the MSC-derived chondrogenesis and to improve the regenerative capacities of MSCs. However, further studies will be necessary to investigate the role of PACAP in these cell cultures.

Additionally, PACAP could be suggested as a new exogenous molecule to improve joint degenerative diseases, since it shows, as seen above, the capacity to prevent cartilage degeneration, as well as chondroprotective properties against various cellular stress conditions such as oxidative stress, mechanical overload, aging process and inflammation, which represent the triggering factors of OA onset. Nevertheless, at present, the role of PACAP in OA development is almost unknown. However, the scarce current evidence concerning the lower PACAP levels found in OA cartilage rat models and in SF of OA patients, seem to suggest that PACAP is closely involved in this pathology (Table 1). Further studies will be needed in the near future for a better understanding of the role of PACAP in articular cartilage and its potential involvement in improving some of the pathophysiological mechanisms involved in OA.

Furthermore, worthy of attention might be also the investigation of the functions of PACAP in the other elements composing the joint, such as the synovial membrane, which actively participates in the maintenance of joint homeostasis and health.

Therefore, as a future perspective, the role of PACAP in chondrogenesis needs to be further investigated in order to have a broader understanding of its functions within the joint structures, and the molecular mechanisms by which it acts. These studies will be paramount to evaluate a possible use of PACAP as a bioactive molecule that could aid in mechanisms of differentiation of mesenchymal stem cells in tissue engineering approaches or in avoiding hypertrophic phenomena in chondrocyte cultures. In addition, future studies on the role of PACAP in physiological and diseased conditions in adult cartilage might be able to investigate the potential therapeutic effects of exogenous PACAP and a possible use of this molecule as a biomarker of degenerative processes and osteoarthritic features, in order to help in identifying pathological stages and tissues involvement

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Conflicts of Interest

The authors declare no conflict of interest.

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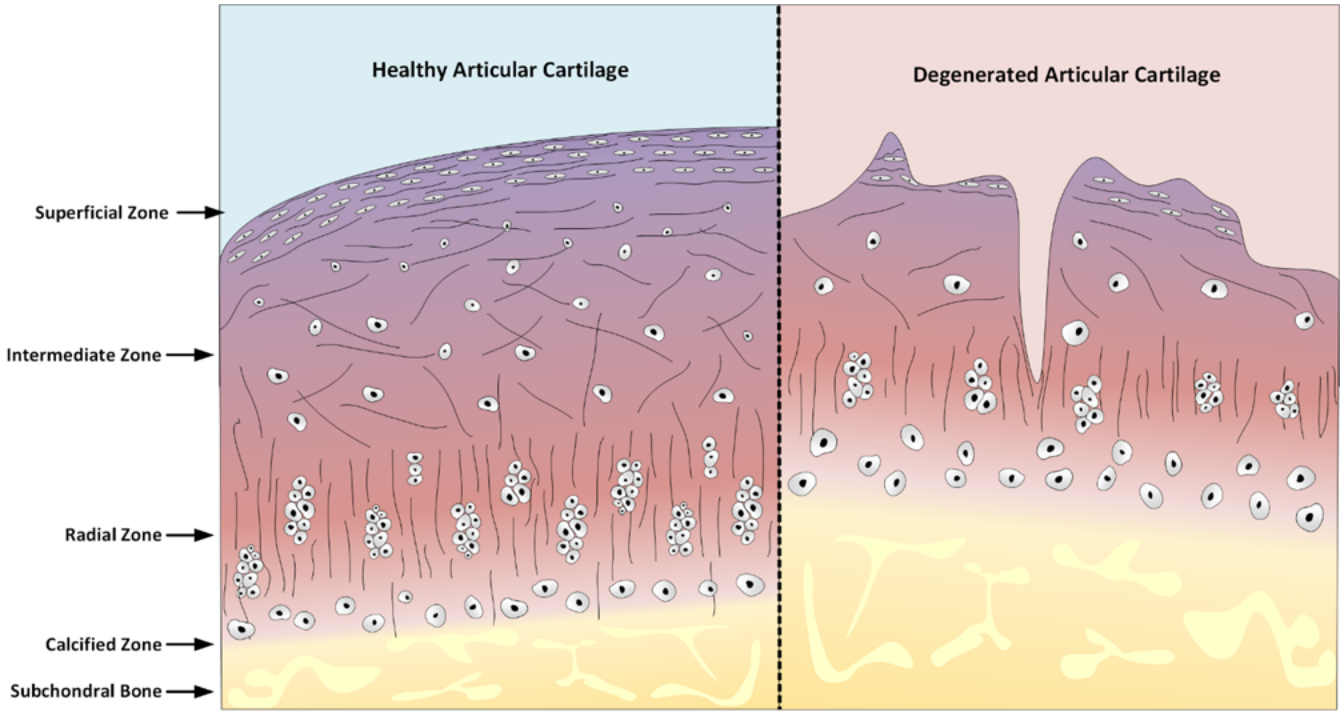


Figure 1. Schematic representation of articular cartilage. On the left, the typical histological organization of a healthy articular cartilage, on the right, a degenerate cartilage. This latter, due to the action of various risk factors, exhibits a destroyed matrix, along with hypocellularity, hypertrophic cells and an increased calcified zone.

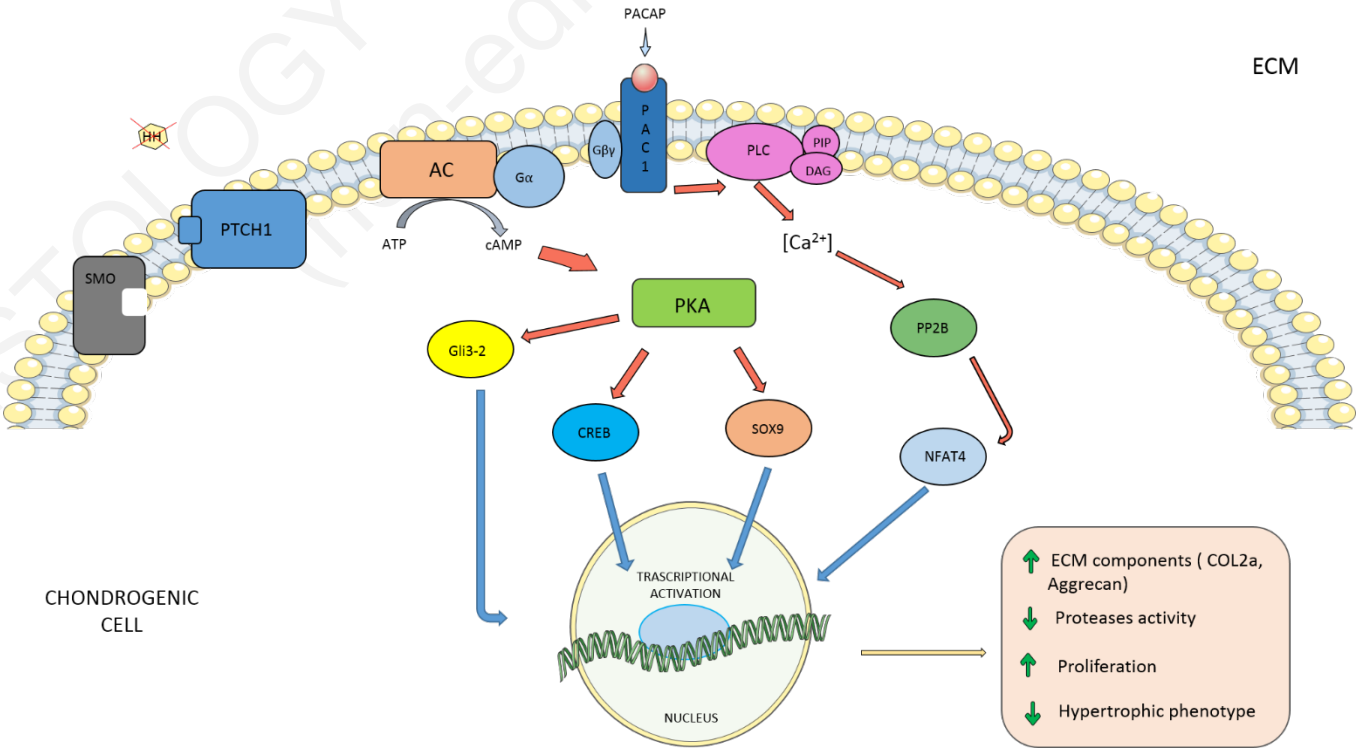


Figure 2. Main signaling pathway activated by PACAP during chondrogenesis. The binding of PACAP with PAC1 increases the cytoplasmic cAMP levels that augment the PKA activity. The latter phosphorylates and activates the transcription factors CREB and SOX9, which migrate in the nucleus and induce the expression of several genes involved in the synthesis of a new ECM and in cellular proliferation and differentiation. Furthermore, PACAP / PAC1 causes an increase in intracellular calcium levels, leading to the activation of PP2B, which activates the NFAT4 transcription factor, also involved in the synthesis of matrix components. Finally, PACAP reduces the levels of HH by blocking its signaling. As a consequence, PKA phosphorylates the transcription factors Gli3 and Gli2, which suppress the transcription of the genes downstream of the HH pathway (Juhàsz et al., 2016).

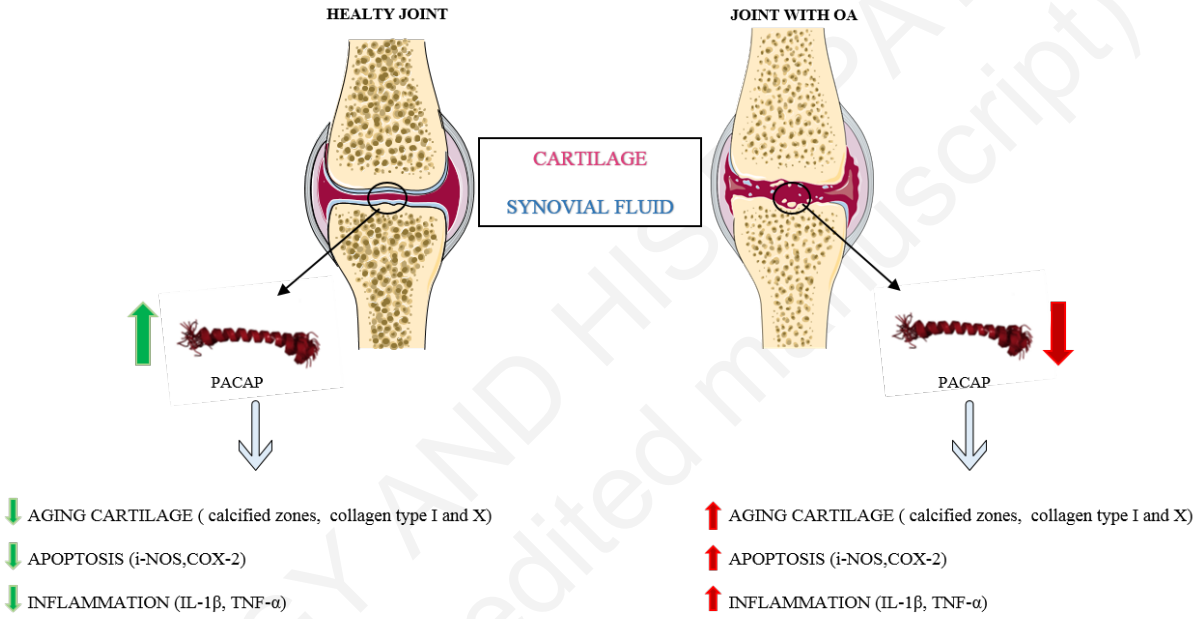
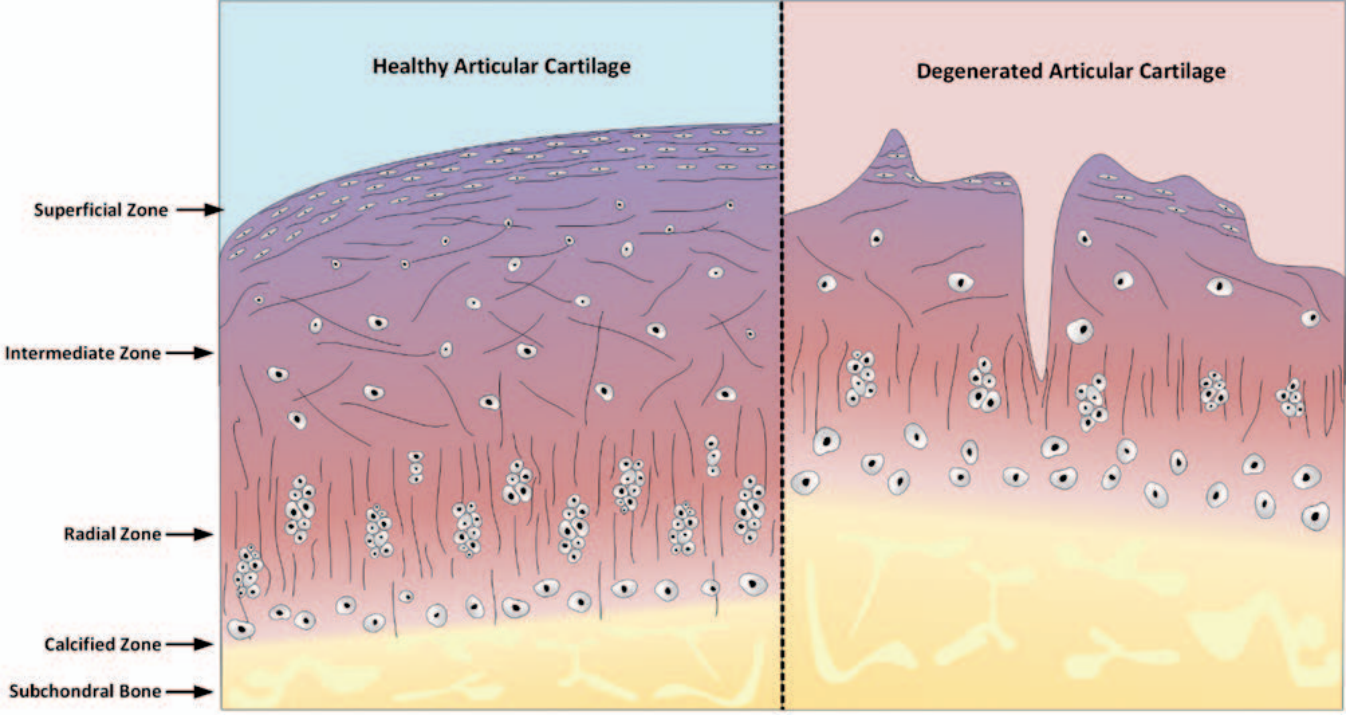


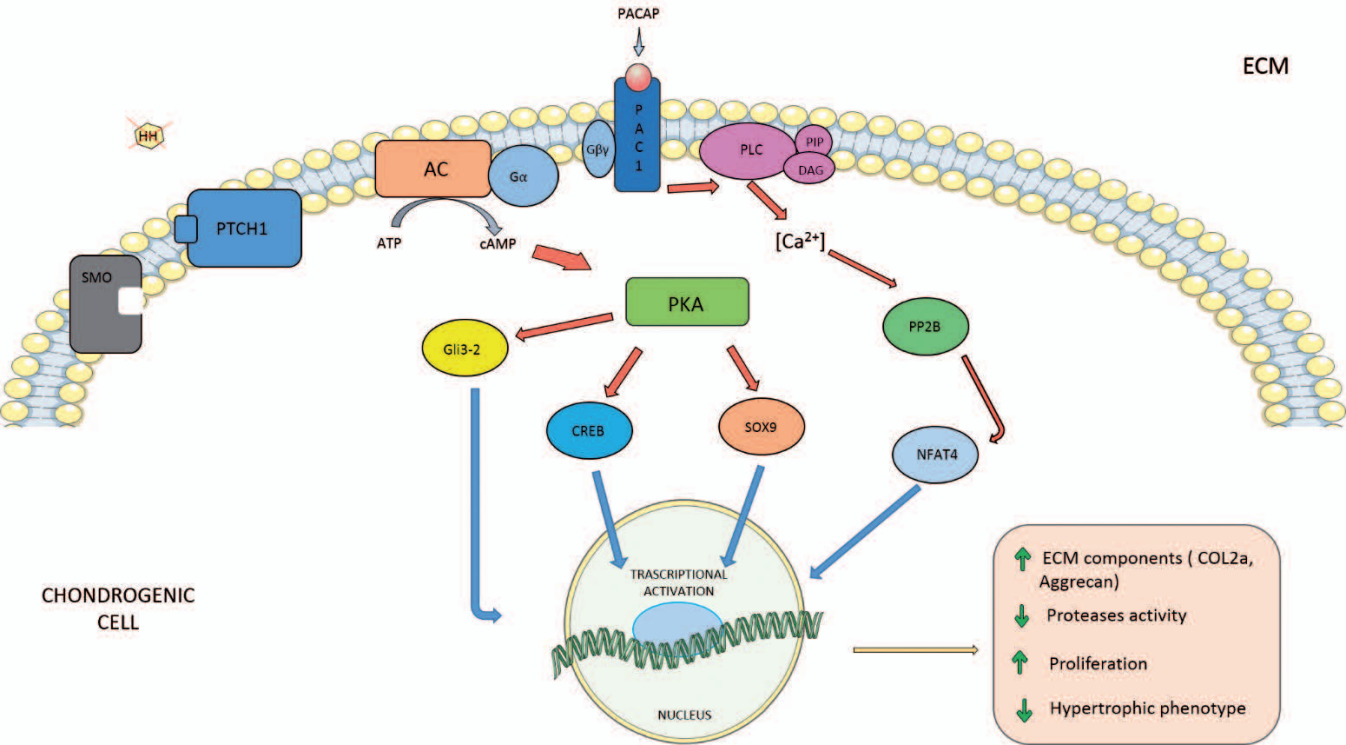
Figure 3: Schematic illustration of PACAP involvement in the joint. The physiological levels of PACAP participate in maintaining the homeostasis of the hyaline cartilage. In OA, PACAP levels in cartilage and SF were found to be lower, compared to healthy controls. Diminished presence of PACAP is associated with an increase in cartilage degeneration and, subsequently, in apoptosis and inflammation processes. The latter is also confirmed by the presence of IL-1β and TNF-α in the SF of OA patients.

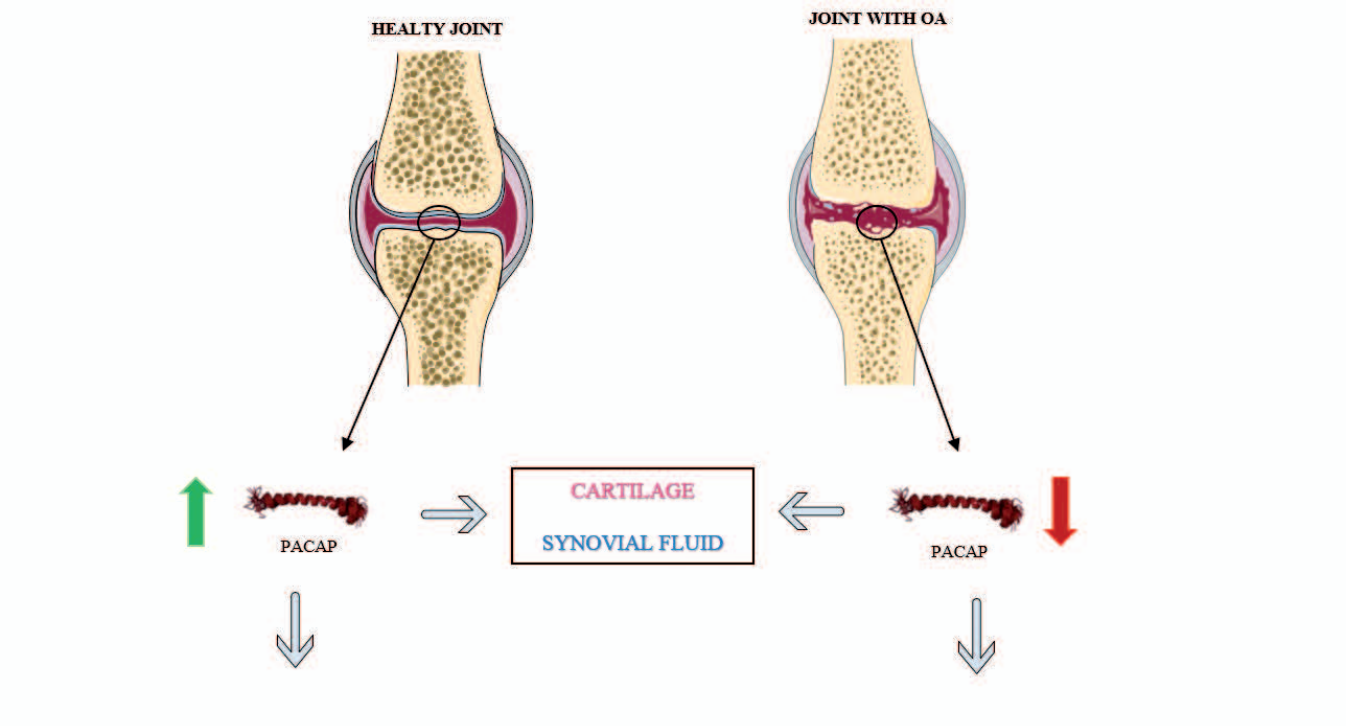
References	Aims	Models	Results
Juhász et al., 2014a	Identify the elements and presumed role of PACAP signaling during chondrogenesis and its possible chondroprotective effects against oxidative stress.	Chicken chondrogenic cells in micromass cell cultures	PACAP and the factors belonging to its pathway (such as PKA, CREB, SOX9, PPB2A) are actively involved in the formation of cartilage, promoting cell proliferation and the production of new ECM. PACAP also protected cartilage formation during H_2O_2-induced oxidative stress.

Juhász et al., 2015b	Investigate the role of PACAP signaling in mechanosensitivity during *in vitro* chondrogenesis.	Chicken chondrogenic cells in micromass cell cultures	PACAP pathway is activated by mechanical stimuli during chondrogenesis. PACAP reduces the hypertrophic effects caused by mechanical stimulus, attenuating the activity of the HH pathway.
Szentléleky et al., 2019	Investigate the involvement of PACAP signaling in the regulation of ECM turnover during *in vitro* chondrogenesis, and under stress conditions in cartilage formation.	Chicken chondrogenic cells in micromass cell cultures	PACAP positively regulates the synthesis of ECM and preserves its integrity, regulating proteases activity. PACAP signaling attenuates the expression and activity of the main proteases such as Hyal2 and Hyal4, ADAMTS4, MMP1, MMP7, MMP8 and MMP13 in cell cultures exposed to oxidative and mechanical stress together
Giunta et al., 2015	Analyse the expression and distribution of PACAP, in the *in vivo* joint elements. Evaluate the PACAP effects on apoptosis and inflammation induced through exposure to increasing concentrations of IL-1β in chondrocyte cultures.	Articular cartilage and synovial fluid from OA rat models. Chondrocyte cultures isolated from healthy articular rat cartilage	PACAP is expressed in cartilage and is present in the SF of healthy rats. PACAP levels in cartilage and SF in OA rat models are lower compared to the healthy rats. PACAP inhibited IL-1β-induced apoptosis, as well as the expression of i-NOS and COX-2 in cultures of isolated chondrocytes.
Szegeczki et al., 2019	Investigate the effects of PACAP genetic suppression, in articular cartilage	Articular cartilage from PACAP KO mice	The lack of PACAP augments articular cartilage aging with OA-like ECM formation, characterised by an increased thickness of the calcified hypertrophic zone along with increased levels of collagen type I and type X. Dysregulation of matrix composition related to decreased expression and activity of PKA/CREB–SOX9 signaling.
Sun et al., 2019a	Investigate the potential correlation of serum and SF levels of PACAP with OA and whether HA injection affects the expressions of PACAP.	Synovial fluid from patients with primary knee OA	PACAP levels in SF but not serum, are significantly lower in OA patients compared with healthy controls. PACAP concentrations were inversely correlated with MMP-3 and IL-1β levels, while higher PACAP levels were detected after HA administration as a treatment in OA.
Sun et al., 2019b	Analyse the PACAP levels in serum and SF in ACL injury patients to determine their relationship with the disease progression of the severity of posttraumatic knee osteoarthritis	Synovial fluid from PTKOA patients with ACL injury	Serum PACAP levels between PTKOA patients and controls have no significant differences. SF PACAP levels are decreased in PTKOA patients compared to the controls. PACAP levels were inversely correlated with levels of IL-1β and TNF-α, suggesting a potential PACAP involvement in inflammatory mechanisms.

Table 1. Summary of studies currently present in scientific literature that analyse the role of PACAP in articular cartilage.


Healthy Articular Cartilage
Degenerated Articular Cartilage
Superficial Zone
Intermediate Zone
Radial Zone
Calcified Zone
Subchondral Bone


PACAP
ECM
HH
PTCH1
SMO
AC
Gα
Gβγ
PAC1
PLC
PIP
DAG
ATP
cAMP
[Ca2+]
PKA
Gli3-2
PP2B
CREB
SOX9
NFAT4
TRASCRIPTIONAL ACTIVATION
NUCLEUS
CHONDROGENIC CELL
ECM components (COL2a, Aggrecan)
Proteases activity
Proliferation
Hypertrophic phenotype



- AGING CARTILAGE (calcified zones, collagen type I and X)
- APOPTOSIS (i-NOS,COX-2)
- INFLAMMATION (IL-1β, TNF-α)

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