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Regulation of senescence associated signaling mechanisms in chondrocytes for cartilage tissue regeneration

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SUMMARY

Adult articular chondrocytes undergo slow senescence and dedifferentiation during in vitro expansion, restricting successful cartilage regeneration. A complete understanding of the molecular signaling pathways involved in the senescence and dedifferentiation of chondrocytes is essential in order to better characterize chondrocytes for cartilage tissue engineering applications. During expansion, cell fate is determined by the change in expression of various genes in response to aspects of the microenvironment, including oxidative stress, mechanical stress, and unsuitable culture conditions. Rapid senescence or dedifferentiation not only results in the loss of the chondrocytic phenotype but also enhances production of inflammatory mediators and matrix-degrading enzymes. This review focuses on the two groups of genes that play direct and indirect roles in the induction of senescence and dedifferentiation. Numerous degenerative signaling pathways associated with these genes have been reported. Upregulation of the genes interleukin 1 beta (IL-1β), p53, p16, p21, and p38 mitogen-activated protein kinase (MAPK) is responsible for the direct induction of senescence, whereas downregulation of the genes transforming growth factor-beta (TGF-β), bone morphogenetic protein-2 (BMP-2), SRY (sex determining region Y)-box 9 (SOX9), and insulin-like growth factor-1 (IGF-1), indirectly induces senescence. In senescent and dedifferentiated chondrocytes, it was found that TGF-β, BMP-2, SOX9, and IGF-1 are downregulated, while the levels of IL-1β, p53, p16, p21, and p38 MAPK are upregulated followed by inhibition of the normal molecular functioning of the chondrocytes. This review helps to elucidate the underlying mechanism in degenerative cartilage disease, which may help to improve cartilage tissue regeneration techniques.

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Introduction

Articular cartilage is a flexible, thin, smooth, connective tissue that covers the surfaces of all of the synovial joints in the human body. Chondrocytes are the primary cellular component of articular cartilage and are required for the production of extracellular matrix (ECM) and for maintaining cartilage structure and function. Articular cartilage injuries due to accidents or trauma affect more than one million people worldwide annually, and surgery is currently the best treatment option. Additionally, osteoarthritis resulting from genetic disorders, obesity, and excessive mechanical loading affects more than 100 million people globally.

Articular cartilage tissue has limited ability to self-repair due to the lack of blood vessels and the paucity of resident progenitor stem cells. Although progenitor cells have been found in different zones of the adult cartilage, the origin and functions of these cells are not completely understood (for review see Candela et al.; Jiang and Tuan). Currently there is a lack of medical treatments that can be used to regenerate articular cartilage. Mediations that aim to re-establish the articular tissue have been developed such as autologous chondrocyte implantation (ACI), marrow stimulation techniques (such as abrasion arthroplasty, drilling, and microfracture), and mosaicplasty.
Treatment via chondrocyte transplantation does not always guarantee a good prognosis because it is difficult to isolate and maintain the normal replicative physiological activity of human chondrocytes in long-term culture. Successful cartilage regeneration or transplantation requires a sufficient quantity of clinical-grade chondrocytes. In vitro monolayer cultures requiring serial cell passages to expand chondrocytes have been extensively adopted for transplantation. The donor age also influences the yield, capacity for expansion in monolayer culture, and redifferentiation ability of chondrocytes. Cell density and proliferation potential of chondrocytes decreases as the age of the donor increases. Barbero et al. reported that chondrocyte yield was similar up to the donor age of 40, and a profound decline was observed in older donors. Additionally, chondrocytes acquired from donors older than 30 years of age exhibited significantly decreased proliferation and showed a greater tendency towards senescence.

Native cartilage architecture has zonal differences in its distribution of ECM. Zonal cells lose specific characteristics during expansion in monolayer culture. In pellet culture, however, deep zone cells produce higher amount of glycosaminoglycans (GAGs) than the superficial zone cells. Surprisingly, another study reported rapid dedifferentiation of the chondrocyte subpopulation after the first passage during monolayer culture and in 3D encapsulation.

When chondrocytes isolated from native cartilage undergo in vitro culture as a monolayer, they lose their phenotype and a variety of senescence and dedifferentiation-mediated genes are turned on. It was found that after four passages, the morphology of chondrocytes changed from polygonal or round to a flattened, amoeboid-like shape, and the cells started synthesizing collagen type X (Col X), as shown in Fig. 1A and B (unpublished data). This result is coincident with previous reports. Senescence and dedifferentiation of chondrocytes during monolayer culture is also accompanied by decreased expression of chondrocyte-specific proteins such as collagen type II (Col II), proteoglycans (PGs), and glycoproteins. The senescence-associated beta-galactosidase assay results shown in Fig. 1C reveal that more senescent cells are observed in late passages compared to early passages (unpublished data). Transplantation of such senescent and dedifferentiated cells results in the formation of undesirable fibrous cartilage at the transplantation site. Loss of chondrocyte-specific properties is due to so-called “stress responses” triggered by in vitro culture conditions. Various kinds of stress such as oxidative, mechanical, damage, aging, and exposure to ionizing radiation are responsible for the senescence of chondrocytes and a large number of genes are involved, e.g., Raf-1/MEK/ERK, p53, and p38 mitogen-activated protein kinase (MAPK). The regulation of genes/transcription factors, which are capable of controlling the native characteristics of chondrocytes using gene therapy, is a promising approach for cartilage regeneration.

This review focuses on the genes required to maintain the normal phenotypic characteristics of chondrocytes as well as direct/indirect roles of senescence and dedifferentiation-mediated genes in chondrocytes and summarizes the correlation between those genes.

Change of chondrocyte phenotype during in vitro culture

Maintaining cellular morphology is important for the regeneration of cartilage tissue and during in vitro monolayer culture chondrocytes show significant phenotypic or morphological changes depending on the culture period. Various proteins are involved in the phenotypic change from polygonal to fibroblastic. The integrity of normal chondrocytes depends on the synthesis of Col II. Various genes related to proliferation, viability, apoptosis, and the synthesis of Col II change as the passage number increases. With increasing passage number, the gene expression of apoptotic or senescent pathways increases while the cell proliferation and viability decreases. For example, passage 2 (P2)
chondrocytes exhibit high expression of Col II and low expression of Col X; however, passage 6 (P6) chondrocytes exhibit low expression of Col II and high expression of Col X [Fig. 1(B)]. In contrast, passage 5 chondrocytes exhibit low expression of Col II and high expression of collagen type I (Col I), indicating a chondrocyte dedifferentiation state. Together with Col II, GAGs also contribute to the mechanical stability of chondrocytes via a structure of an interwoven meshwork. Another core protein, aggrecan, plays an important role in maintaining the chondrocyte phenotype by linking covalently with GAGs, resulting in the production of matrix PGs. This complex model of ECM involving Col II helps chondrocytes to maintain vital interactions with one another. Therefore, cells deprived of ECM molecules rapidly undergo senescence or apoptosis.

Chondrocytes undergoing senescence actively produce cartilage-degrading enzymes such as matrix metalloproteinase 13 (MMP13) and aggrecanases, which promote the degradation of aggrecan and degrading enzymes such as matrix metalloproteinase 13 (MMP13) of chondrocytes are not well-understood. Another approach to molecular mechanisms involved in the process of re-differentiation is still not clear, but its expression in chondrocytes is considered the key for recovery of chondrogenic properties. To recover chondrogenic properties, a number of senescence-related genes have been applied to chondrocytes in vitro without significant disruption in the cellular morphology.

Several alternatives to conventional monolayer culture have been explored including growth factor treatment, pellet culture, and bioreactor treatment. Techniques such as functionalization of the dynamic culture surfaces with ECM, culture on a continuously expanding surface, and surface coating with Col I have shown great potential for inhibiting dedifferentiation and recovering chondrogenicity. Other reports have also revealed the role of mechanical loading and shear stress in the regulation of phenotypic characteristics of chondrocytes during in vitro culture. Alternately, excessive mechanical loading and high fluid shear also recapitulated the genes associated with OA. Most importantly, these treatments were relatively effective in maintaining the phenotypic characteristics of chondrocytes but sufficient expansion of chondrocytes was not achieved. According to Manoil et al., a twenty-fold minimum multiplication of donor chondrocytes usually obtained is required to recover the average cartilage defect of 4 cm³. Chondrocyte expansion is critical for clinical applications and depends on multiple factors including the site of biopsy, cell seeding density, supplements in the culture medium, and the cultivation period.

Despite promising outcomes using these various techniques, the molecular mechanisms involved in the process of re-differentiation of chondrocytes are not well-understood. Another approach that has been widely studied is the delivery of genes related to growth factors, i.e., transforming growth factor beta-1 (TGF-β1), BMP, insulin-derived growth factor (IGF), and fibroblast growth factor (FGF); transcription factors, i.e., SRY (sex determining region Y)-box 9 (SOX9), RUNX2, and SMAD; microRNAs, and Col II promoter-binding proteins.

We believe this could be a novel approach for obtaining a sufficient quantity of normal chondrocytes, but further studies are required to sort out common genes that control most of the signaling pathways involved in senescence. Further in this review, we focus on the multiple genes that are directly or indirectly involved in senescence and try to determine common genes that control multiple pathways.

**Genes required for the maintenance (lineage determination) of chondrogenic properties and are indirectly involved in senescence**

**Transforming growth factor-beta (TGF-β)**

TGF-β is a cytokine and plays an important role in the induction of chondrogenesis. There are three isoforms of TGF-β, and among them TGF-β1 has been the most widely studied in chondrocytes. TGF-β1 follows a complex signaling mechanism that ultimately helps to maintain the chondrocytic characteristics during in vitro culture via promoting cell proliferation, ECM protein synthesis, and protecting normal morphology by inhibiting MMPs.

Since Skonier et al. isolated the TGF-β gene from human adenocarcinoma cells and identified the function of the TGF-β protein, it has been reported that many cell types, including chondrocytes, produce TGF-β as a high-molecular weight macro-molecule in association with the latent homodimer with latent TGF-β-binding protein (LTBP). The LTBP complex mediates the deposition of the latent complex to the ECM. Release of the mature TGF-β from the latent complex is a multifaceted process involving proteolytic cleavage and deglycosylation of latency associated peptide (LAP)44. In chondrocytes, TGF-β signaling involves the heterotetrameric complex including two type I receptors also known as activin receptor like kinases (ALKs) and two type II receptors (TβRII)45. The type I receptors are ultimately phosphorylated by type II receptors when dimeric TGF-β molecules bind to the tetrameric complex46. ALK5 activation is crucial in TGF-β signaling that leads to phosphorylation of SMAD2 and SMAD347. TGF-β1 promotes proliferation of chondrocytes through β-catenin signaling48 and maintains the polygonal phenotype of chondrocytes by enhancing Col II synthesis through SMAD3 activation of SOX9 together with the SMAD2 and SMAD4 complex (Fig. 2)49. The SMAD2/3 signaling pathway also inhibits maturation and hypertrophy of chondrocytes during in vitro culture. Mice with Smad3 deficiency develop cartilage degenerative disease and those with TGF-β1 deficiency develop osteoarthritis50–52.

TGF-β1 has variable roles in chondrogenesis in vivo and in vitro. Introducing TGF-β1 into the peristium of a femur causes chondrocyte differentiation and cartilage formation, while TGF-β1 inhibits and slows down chondrocyte maturation (hypertrophy) in vitro53,54. TGF-β1 is a major regulator of molecular and physiological characteristics of chondrocytes. Therefore, in the TGF-β1 signaling pathway, any gene inhibiting TGF-β1 may result in senescence and dedifferentiation.

**BMPs**

BMP signaling pathways are also crucial regulators of chondrogenesis and potent inducers of cartilage formations. More than 20 types of BMP-related proteins comprising the BMP family have been described, which are categorized into three main groups depending on their function and sequence homology: the BMP2/4 group, the osteogenic protein 1 (OP1, BMP7) group, and the growth and differentiation factor 5 (GDF5) group. BMP-2 is a cytokine required for in vitro chondrocyte culture. Like TGF-β1, BMP-2 follows a complex downstream signaling mechanism for the activation and transcription of target genes. BMPRIA (also known as ALK3), BMPRIB (also known as ALK6), and various SMADs are activated by BMP-2, expressed independently, and essential for in vitro maintenance of chondrocyte morphology, in vivo chondrogenesis, and cartilage growth development. Combinational therapy of BMP-2 with BFGF in three-dimensional culture significantly enhances the expression of Col II and aggrecan; however, similar treatments to monolayer culture result in no effect on Col II and
aggrecan. BMP-2 repairs damaged cartilage via boosting PG synthesis. BMP-4, -6, -7, -9, and -13 also help to maintain chondrocytes and induce chondrogenesis of MSCs in vitro. However, there is still debate about the relative potencies of BMPs due to variation in the model systems and culture conditions used.

SOX9

SOX9 is a transcriptional regulator for chondrogenesis confirmed by multiple in vitro and in vivo studies on mice and humans. SOX9 also has a significant role in mesenchymal condensation following chondroblast differentiation. Genetic studies using Sox9−Ires−Cre knock-in mouse models showed that SOX9-presenting cells are the progenitors of chondroblasts. Recently, mouse genetic analysis revealed that Wnt/β-catenin signaling plays a strong role in the regulation of both the Sox9 and Runx2 genes and, in particular, Wnt signaling suppresses chondrogenic differentiation while increasing osteoblastic differentiation. In addition to Wnt signaling, Notch signaling and HES-1 are potent negative regulators of SOX9 that ultimately lead to decreased expression of Col II. SOX9 is ultimately activated by almost all the growth factors associated with chondrogenic lineage maintenance. Furthermore, SOX9 alone can regulate almost all of the genes required for COL II, core protein, and aggrecan production.

Insulin-like growth factor-1 (IGF-1)

IGF-1 is one of the significant growth factors present in cartilage and has a unique endocrine, paracrine, and autocrine signaling mechanism. Principally, IGF-1 increases cell proliferation, enhances synthesis of matrix-associated proteins, and inhibits apoptosis by regulating downstream signals via phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK), a member of the MAPK cascade. PI3K activates cell survival and protein synthesis through downstream signaling of Akt and p70S6 kinase, respectively. On the other hand, the ERK signaling cascade is engaged in downstream activation by RAS (mostly stimulated by IGF-1) and growth-factor-receptor bound protein 2 (GRB2). IGF-1 stimulates PG synthesis in human articular chondrocytes during in vitro culture by activating the PI3K/Akt/mTOR/p70S6 kinase pathway. PG synthesis, as well as Akt phosphorylation was inhibited by using the IGF-1 antagonist. Significant downregulation of PG synthesis was observed when mTOR and p70S6 kinase were inhibited.

SOX9 is also involved in the redifferentiation of chondrocytes via a novel post-translational regulatory mechanism. SOX9 is able to re-express chondrogenic markers even in degenerated chondrocytes, implying that SOX9 has great potential to recover chondrogenic function in degenerated cartilages. As shown in Fig. 1D, early-passage (P2) degenerated chondrocytes isolated from damaged cartilage show similar fibroblastic morphology to late-passaged chondrocytes (P6), and these degenerated chondrocytes also exhibit higher expression of COL I compared to normal chondrocytes [Fig. 1(E)]. SOX9 is ultimately activated by almost all the growth factors associated with chondrogenic lineage maintenance. Furthermore, SOX9 alone can regulate almost all of the genes required for COL II, core protein, and aggrecan production.

**Fig. 2. Genes involved in the maintenance of normal phenotypic chondrocytes and can regenerate cartilage.** Chondrocytes isolated from normal articular cartilage can regenerate cartilage again after expansion in monolayer culture if followed the signaling mechanism of IGF-1, TGF-β and BMP-2. IGF-1, TGF-β and BMP-2 ultimately involved in the formation of GAG matrix required for maintaining chondrocytes normal phenotype and molecular functions. Inhibition or down regulation of IGF-1, TGF-β, BMP-2 and especially Sox9 cause senescence in the chondrocytes. SOX9 seems to be a major regulator of controlling senescence in chondrocyte via maintaining chondrocytes normal phenotype/morphology through Col2a1, core protein and aggrecan (For further details please see the text).
Thus, IGF-1 is involved in the activation of the PI3K/Akt pathway followed by the mTOR and p70S6 kinase pathways (Fig. 2).

In chondrocytes, the primary role of IGF-1 in PG synthesis is to stimulate translational activity rather than transcription activity\textsuperscript{70–72}. IGF-1 induces the differentiation of mesenchymal cells into chondrocytes through the PI3K pathway as opposed to the ERK pathway because inhibition of ERK is not able to block the synthesis of collagen and PG\textsuperscript{71}.

In chondrocytes, the pathway of IGF-1 to PI-3 kinase to Akt signaling also promotes matrix synthesis (including Col II) and survival through increased SOX9 expression\textsuperscript{70}. Recently, it has been observed that stress in the endoplasmic reticulum stimulates the expression of tribble homology 3 (TRB3), and TRB3 is also known as a strong inhibitor of Akt. TRB3 inhibits Akt phosphorylation, and the resulting stress leads to a decrease in the survival of chondrocytes\textsuperscript{73}. Induction of oxidative stress in normal chondrocytes resulted in the same change in signaling pathway that is found in degenerated nucleus pulposus chondrocytes (dNPCs) isolated from osteoarthritis patients [Fig. 1(F)], presumably because of inhibition of the IGF-1 pathway via Akt inhibition. dNPCs also show low GAG matrix formation as shown by alcian blue staining when compared to normal NPCs [Fig. 1(G)]. These findings have also been observed in normal chondrocytes undergoing oxidative stress\textsuperscript{5,18}. Figure 2 illustrates the brief signaling pathway of IGF-1 in chondrocytes.

Genes directly involved in cellular senescence and dedifferentiation of chondrocytes

Oxidative stress or reactive oxygen species (ROS) are considered a major regulator of senescence in chondrocytes. ROS activate multiple genes that induce senescence, dedifferentiation, and even apoptosis via initiation of multiple downstream signals as shown in Fig. 3. The genes and their interconnection pathways are discussed in detail as follows.

**Interleukin 1 beta (IL-1β)**

The cytokine IL-1β works as a negative regulator for chondrogenicity, inhibits proliferation, and often induces dedifferentiation in chondrocytes. There is direct evidence that there is an increase in the IL-1β level, associated with dedifferentiation, during monolayer expansion of human chondrocytes after four passages\textsuperscript{74}. IL-1β-associated dedifferentiation of chondrocytes involves multiple mechanisms such as the repression of SOX9 and VEGF and induction of nitric oxide (NO) synthase and MMPs\textsuperscript{75}. IL-1β activates B-Raf in turn causing MEK1/2 and ERK1/2 phosphorylation, but fails to induce expression of SOX9 and VEGF, and inhibited the proliferation of articular chondrocytes\textsuperscript{76}. IL-1β induces aggrecan degradation by activating aggrecanase through the MAPK and ERK1/2 signaling mechanism. IL-1β is also associated with the induction of other signals such as phosphorylation of p38 and activating transcription factor-2 (ATF-2), C-JNK, c-fos, activating protein (AP-1), and nuclear factor kappa B (NF-κB), that ultimately activates aggrecanase\textsuperscript{77}. IL-1β is also associated with the induction of NO synthase, which has the ability to activate JNK, and JNK is a well-known inducer of senescence and mediator of osteoarthritis\textsuperscript{78}.

As IL-1β induces dedifferentiation of chondrocytes, SOX9 and Col II are downregulated through α-, β-, and γ-catenin in the Wnt signaling pathway\textsuperscript{79}. The inhibition of catenin signaling induced by IL-1β via a low dosage of γ-radiation can inhibit dedifferentiation and inflammation of chondrocytes\textsuperscript{80}. Chondrocytes with high expression of IL-1β may undergo apoptosis because IL-1β enhances...
ROS and p53. Chondrocytes treated with IL-1β for 5 days in vitro became senescent and exhibited a decrease in expression of aggrecan with upregulation of p16\(^{34}\). On the other hand, inhibition of IL-1β with resveratrol decreased p53 and inhibited apoptosis\(^{32}\). Moreover, in dedifferentiated chondrocytes, IL-4 induced catabolic events such as NO production, a release of collagenase by down-regulating IL-1β, and increased proliferation of chondrocytes\(^{35}\). Further, ERK1/2, also involved in IL-1β, enhanced MMP3 and MMP13 expression and inhibited COL II and aggrecan expression in chondrocytes. Inhibition of either ERK1 or ERK2 reverted the altered gene expression of MMP13, MMP3, COL II, and aggrecan induced by IL-1β in human chondrocytes\(^{34}\).

**p53, p16, and p21**

In chondrocytes, two different mechanisms of senescence have been suggested, including replicative senescence and stress-induced premature senescence (SIPS). Replicative senescence is due to telomere shortening, while the mechanism for SIPS may involve oxidative stress and DNA damage without a change in telomere length\(^{36},37\). p53, p21, and pRB genes follow the telomere shortening pathway to induce senescence as well as apoptosis in chondrocytes. However, p16 and pRB play a synergistic role in senescence through SIPS\(^{38}\). In senescent cells, the expression of p53, p21, p16, and pRB increases and results in cell cycle arrest through the inhibition of multiple cyclin-dependent kinases. Articular chondrocytes with high expression of p21, p16, and pRB also exhibit more positive senescent cells with SA-βgal staining\(^{37}\). Additional studies reveal that NO increases the expression of p53 by phosphorylating p38 MAPK and p53, further upregulating RAX, a pro-apoptotic gene\(^{38}\). Chondrocytes expressing p53 show similar morphology to osteoarthritic cartilage cells and exhibit apoptosis, whereas downregulation of p53 expression can prevent chondrocytes from undergoing apoptosis or senescence\(^{39}\). A more important gene associated with senescence is p16, and chondrocytes expressing p16 start losing the normal phenotype rapidly, expressing more inflammatory cytokines (IL-1β and IL-6) and MMPs (MMP1 and MMP13)\(^{34}\). ROS are direct mediators of p16 that promote senescence and dedifferentiation\(^{40}\). Mainly, p16 engages in cell cycle arrest at the G1 stage by blocking CDK4/6 and main-}

**Future research prospective**

In the last decade, the major goal for researchers has been to develop novel therapeutic strategies for cartilage defects. Existing therapeutic treatments are symptomatic and only alleviate the pain instead of controlling disease progression. Recently, ACI has been considered a successful treatment. Therefore, to develop effective therapies, an understanding of the molecular signaling mechanisms of chondrocytes is essential. For autologous transplantation, immune rejection is not the major obstacle but rather the lack of availability of large numbers of chondrocytes and maintaining chondrocyte viability during in vitro expansion is a deterrent.

There are numerous genes responsible for the direct induction of senescence, which also change the phenotypic and molecular characteristics of chondrocytes. Among them, IL-1β and p38 MAPK are the most critical genes which play direct role in the induction of senescence. Similarly, there are certain genes that induce senes-}

**Conclusion**

Despite numerous studies on chondrocytes and cartilage regeneration, a comprehensive understanding of chondrocyte behavior, growth, death, matrix remodeling, and interaction between signaling pathways is lacking. Multiple complex signaling pathways are involved in the senescence and dedifferentiation of chondrocytes, therefore, a systematic approach is needed to sort out the key genes exhibiting crucial roles in regulating the fate of chondrocytes. From the review of numerous studies, it has been made clear that oxidative stress is one of the major factors in the induction of senescence in chondrocytes through multiple signaling pathways. Specifically, oxidative stress involved in the degeneration of chondrocytes occurs via upregulation of IL-1β and p38 MAPK. On the other hand, down regulation of SOX9 significantly...
reduces the chondrocytes' cartilage tissue regeneration potential. If we can control the expression of SOX9, IL-1β, and p38 MAPK, then senescence and dedifferentiation in chondrocytes can be overcome to regenerate proper cartilage tissue. Signaling pathways and molecular processes are complex; therefore, further in vitro and in vivo animal studies are required to achieve our goal of controlling senescence in chondrocytes by manipulating a single master gene.

**Authors contributions**

The idea of this study was conceived and brought in to write up form by Sajjad Ashraf. All other authors contributed to make the final form of manuscript. Byung-Hyun Cha helped to draw the figures used in the manuscript. Jin-Su Kim and Jongchan Ahn contributed for the experimental data used as an example to strengthen the study. Inbo Han and Hansoo Park critically reviewed and edited the manuscript. Whole study was done under the supervision of Soo-Hong Lee.

**Author disclosure statement**

No competing financial interests exist.

**Conflicts of interest**

The authors have declared that there is no conflict of interest.

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