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Alexandre C Dufour, Marie Buffier, Delphine Vertu-Ciolino, François Disant, Frédéric Mallein-Gerin, et al.. Combination of bioactive factors and IEIK13 self-assembling peptide hydrogel promotes cartilage matrix production by human nasal chondrocytes. *Journal of Biomedical Materials Research Part A*, 2019, 107 (4), pp.893-903. 10.1002/jbm.a.36612 . hal-02199847

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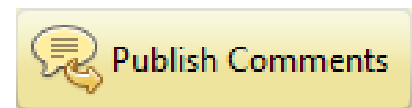
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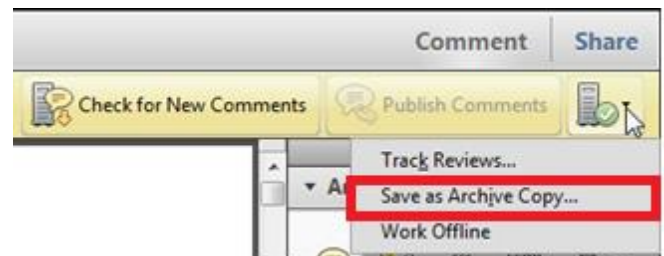
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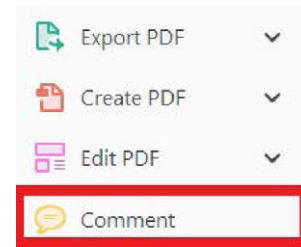
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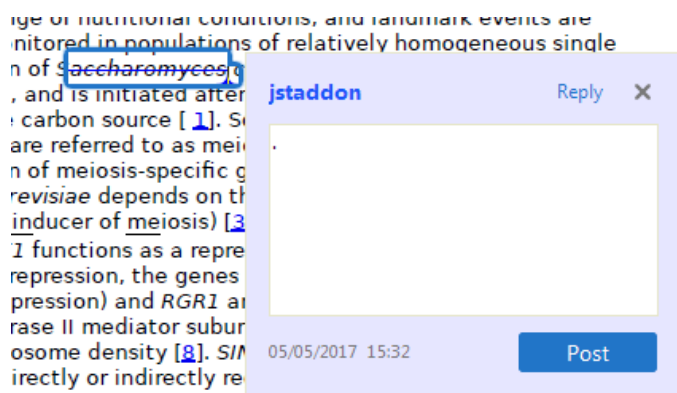


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
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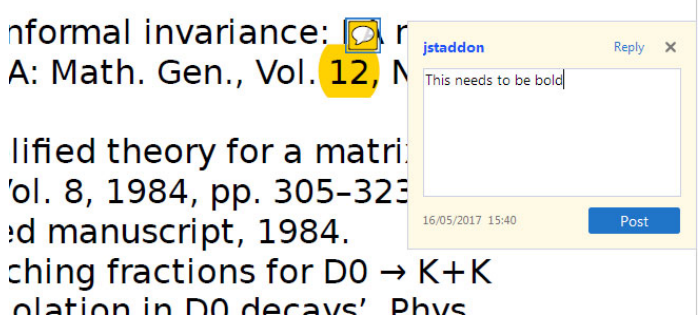
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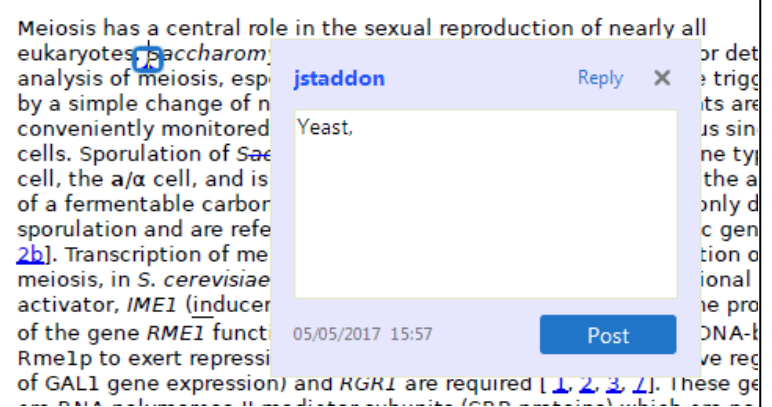


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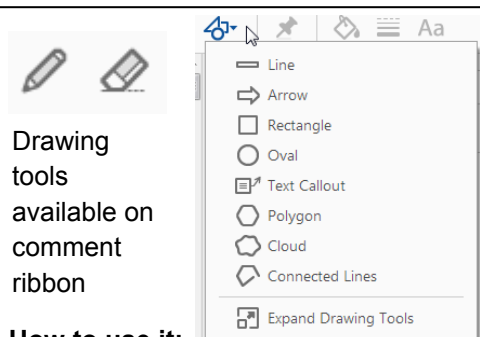
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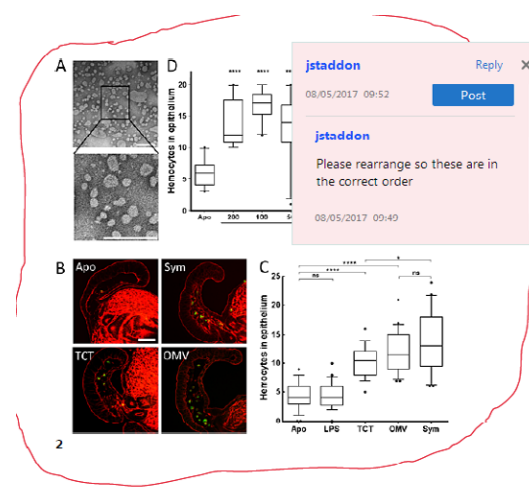


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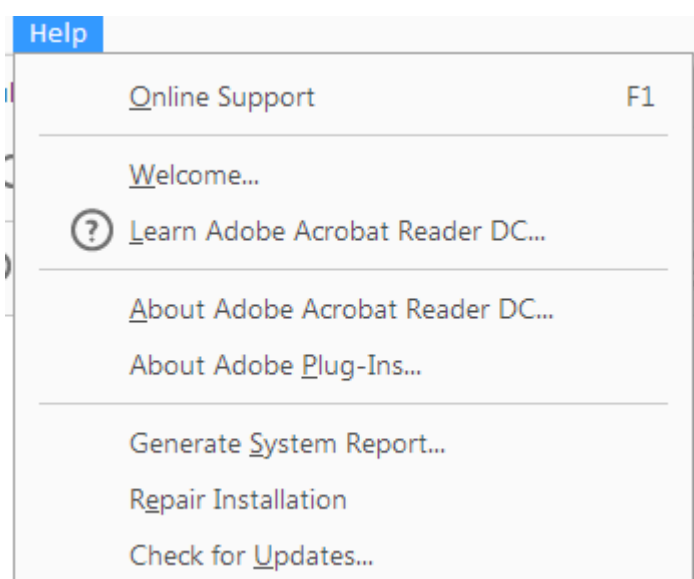
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Combination of bioactive factors and IEIK13 self-assembling peptide hydrogel promotes cartilage matrix production by human nasal chondrocytes

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Received 17 October 2018; revised 17 December 2018; accepted 9 January 2019

Published online 00 Month 2019 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.36612

Abstract: Nasal reconstruction remains a challenge for every reconstructive surgeon. Alloplastic implants are proposed to repair nasal cartilaginous defects but they are often associated with high rates of extrusion and infection and poor biocompatibility. In this context, a porous polymeric scaffold filled with an autologous cartilage gel would be advantageous. In this study, we evaluated the capacity of IEIK13 self-assembling peptide (SAP) to serve as support to form such cartilage gel. Human nasal chondrocytes (HNC) were first amplified with FGF-2 and insulin, and then redifferentiated in IEIK13 with BMP-2, insulin, and T3 (BIT). Our results demonstrate that IEIK13 fosters HNC growth and survival. HNC phenotype was assessed by RT-PCR analysis and neo-synthesized extracellular matrix was characterized by western blotting and

immunohistochemistry analysis. BIT-treated cells embedded in IEIK13 displayed round morphology and expressed cartilage-specific markers such as type II and type IX collagens and aggrecan. In addition, we did not detect significant production of type I and type X collagens and gene products of dedifferentiated and hypertrophic chondrocytes that are unwanted in hyaline cartilage. The whole of these results indicates that the SAP IEIK13 represents a suitable support for hydrogel-based tissue engineering of nasal cartilage. © 2019 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 00A: 000–000, 2019.

Key Words: chondrocyte, nasal cartilage reconstruction, cartilage tissue engineering, self-assembling peptide

How to cite this article: Dufour A, Buffier M, Vertu-Ciolino D, Disant F, Mallein-Gerin F, Perrier-Groult E. 2019. Combination of bioactive factors and IEIK13 self-assembling peptide hydrogel promotes cartilage matrix production by human nasal chondrocytes. *J Biomed Mater Res Part A* 2019:9999A:1–11.

INTRODUCTION

Nose is the primary portal of entry for the respiratory system in human. It acts as an air conditioner and a defender of the lower respiratory tract by filtering, humidifying, and heating the inhaled air. It also serves as the major sensory organ for smell. Besides these physiological functions, the nose plays a critical role in harmony and radiance of the face because of its central position. The nasal cavity is divided into two by a key cartilage structure, the nasal septum. In case of defect, nasal cartilage is not able to regenerate because of its lack of vasculature and isolation from systemic regulation, and thus nasal reconstruction remains a challenge. Autologous or homologous

grafts are used to repair cartilaginous defects of the nose but availability, donor site morbidity, and technical difficulty implying long operative time limit autologous grafts. Issues have been raised with homologous grafts such as risk of infection and long-term resorption.¹ The use of alloplastic implants represents an interesting alternative but despite advances in research with alloplastic materials, they are still associated with risks of extrusion and infection^{2–4} and poor biocompatibility.^{4,5} In this context, the use of a porous scaffold filled and coated with an autologous cartilage gel would be advantageous. Porous polymers are already used for nasal reconstruction, mainly for aesthetic reasons.⁶ Regarding gels, self-assembling peptides

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Contract grant sponsor: Centre National de la Recherche Scientifique

Contract grant sponsor: Fondation de l'Avenir pour la Recherche Médicale Appliquée; contract grant number: AP-RMA-2015-007

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(SAPs) have recently emerged as a class of next-generation hydrogels for tissue engineering applications. These synthetic materials consist of short peptide sequences that are able to self-assemble and form a tridimensional network that mimics extracellular matrix (ECM) environment when peptide concentration and physical conditions are adequate.⁷⁻¹² The peptide sequence is composed of alternating hydrophilic and hydrophobic amino acids that form stable β -sheet structures in deionized water and self-assemble into interweaving nanofibers when exposed to an electrolyte solution such as cell culture medium.⁷⁻¹² In contrast to other natural material-based hydrogels such as collagen, fibrin, alginate, or hyaluronan, advantages of such synthetic material include low risks of biological pathogen transmission and homogeneous batches in production.^{13,14} Moreover, studies have shown that SAPs do not elicit immunogenic or inflammatory response *in vitro*¹⁵ or *in vivo*.^{10,16}

In the specific context of cartilage repair, SAPs are interesting materials since the self-assembling process results in highly hydrated material (more than 90% water content)¹⁷ and native cartilage is highly hydrated (80% water content),¹⁸ a feature offering the tissue its "shock absorber" capabilities. SAPs also offer the advantage of being injectable into nonuniform defects,¹⁹ thanks to their malleability and it is highly conceivable that they can be used to infiltrate and coat porous scaffolds intended to serve as implants. Moreover, SAPs have been shown to sustain or allow recovery of the chondrogenic phenotype with expression of cartilage-specific matrix proteins when they are combined with rabbit,²⁰ bovine,²¹⁻²⁴ and human²⁵ chondrocytes. Therefore, SAPs appear as promising supports to form cartilage gel. Following this view, the aim of this work was to evaluate if a new SAP sequence, referred as IEIK13, could provide a suitable three-dimensional environment for chondrogenic expression of human nasal chondrocytes (HNC). We used cocktails of specific factors to expand the cells on plastic (fibroblast growth factor (FGF)-2 and insulin, designated FI) and to allow their redifferentiation in IEIK13 hydrogel (bone morphogenetic protein (BMP)-2, insulin, and triiodothyronine T3, designated BIT). This sequential addition of factors has been proven efficient to amplify human chondrocytes isolated from diverse anatomical sites (including nose) and to induce their redifferentiation in collagen²⁶ or agarose²⁷ gels and in collagen sponges.^{28,29}

MATERIALS AND METHODS

Cell extraction and amplification

Primary cultures of HNC were prepared from healthy septal cartilages of 3 donors (age range: 21-45) undergoing rhinoplasty. Nasal cartilage samples were collected upon written informed consent of donors and complied with local ethics guidelines, national and European Union legislation regarding human sample collection, manipulation, and personal data protection (Ethics Committee for research with human samples, CODECOH: DC-2014-2325). For chondrocyte extraction, septal cartilages were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich) supplemented with 50 μ g/mL streptomycin (Panpharma) then cut in small slices and digested overnight at 37°C with 0.5 mg/mL bacterial

collagenase A (Roche Applied Science) in culture medium composed of Dulbecco's modified Eagle medium/Ham's F12 (DMEM-F12) (Gibco/Life technologies). Chondrocytes were then seeded on culture dishes at a density of 1.5×10^4 cells/cm² in culture medium supplemented with 50 μ g/mL streptomycin and 10% fetal bovine serum (FBS) (Gibco-invitrogen). At this stage, cells were designated P0. Forty-eight hours after seeding, the medium was refreshed and further supplemented with 5 ng/mL fibroblast growth factor (FGF)-2 (R&D Systems) and 5 μ g/mL insulin (Umuline Rapide, Lilly). The combination of FGF-2 and insulin was referred as FI. FI-supplemented complete medium was replaced three times a week. The cells were cultivated for 2 weeks and then trypsinized.

Cell culture in IEIK13 self-assembling peptide hydrogel

IEIK13 peptide solution was provided by 3-D Matrix, Inc. (Needham, MA). Peptide sequence is Ac-IEIKIEIKIEIKI-NH₂ [Fig. 1(A)]. Peptide powder was synthesized by CPC (Chinese Peptide Company, Hangzhou, China) and was dissolved in 10% wt/vol sucrose solution at a peptide concentration of 1.5% (wt/vol). Passaged P0 chondrocytes were resuspended in a volume of 10% wt/vol sucrose solution then mixed with a volume of the 1.5% peptide solution so that the final peptide solution was 1% (wt/vol) and the cell density was 2×10^6 cells/mL. The cell-peptide solution was then carefully homogenized by reverse pipetting and 100 μ L of gel was poured in wells of a 48-well culture plate. One milliliter of DMEM-F12 medium was then carefully added to each well and the resulting mixed solutions were incubated at 37°C for 1 h to allow self-assembling. Finally, 500 μ L of culture medium was substituted by new medium so that the complete medium contained 1 \times ITS (insulin, transferrin, and selenium, Gibco/Life technologies), 50 μ g/mL 2-phospho-L-ascorbic acid (trisodium salt, Fluka) supplemented or not with 200 ng/mL of recombinant human bone morphogenetic protein (BMP)-2 (Dibotermine- α , drug form of BMP-2 contained in InductOs kit, Wyeth), 5 μ g/mL insulin (Umulin, Lilly), and 100 nM T3 (triiodothyronine, Sigma). The combination of BMP-2, insulin, and T3 was referred as BIT. The culture medium without BIT was designated as control medium (CTRM). The medium was replaced by half every 2 days over a culture period of 21 days.

Evaluation of cell growth

After 7, 14, and 21 days of culture in IEIK13 hydrogel in the presence of CTRM, 1 mL PBS was added to the wells and cells were mechanically dissociated from hydrogel by several reverse-pipetting then centrifuged. Following PBS removal, cell-matrix interactions were further disrupted with trypsin and cells were stained with Trypan blue and counted with a Cellometer (Nexcelom). For each time point, the rate of growth was calculated over the entire culture period using the following formula: growth rate = $(\ln(T/S)/\ln(2))/D$, where S is the number of cells initially seeded (in our case 200,000 cells for a 100 μ L gel), T is the number of cells obtained after trypsinization, and D is the number of days in culture.

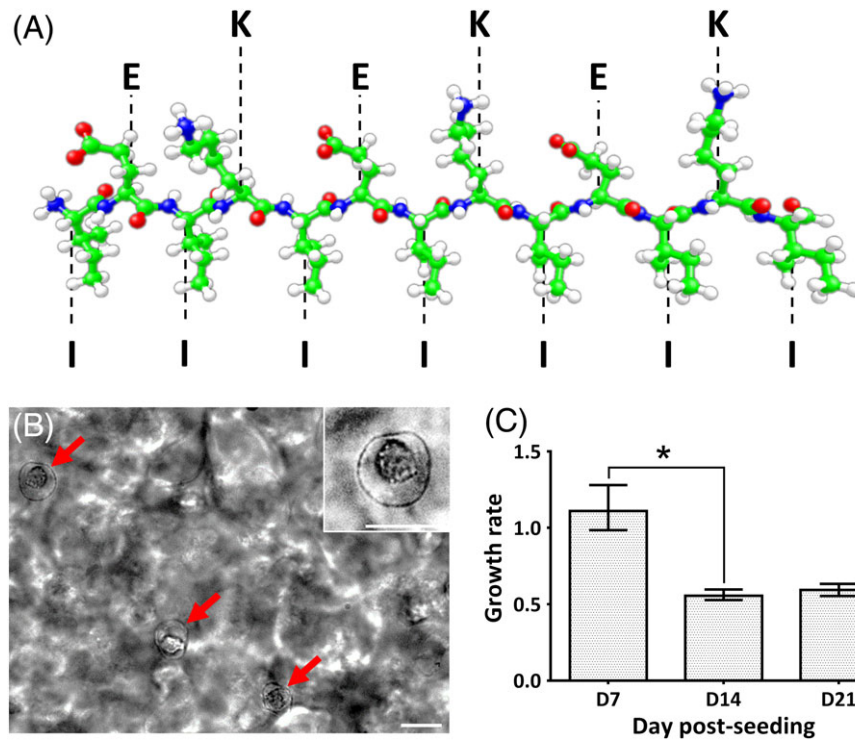


FIGURE 1. A: Molecular representation of a single IEIK13 self-assembling peptide. The peptide sequence AcN-IEKIEKIEKI-CNH2 referred as IEIK13 comprises alternating hydrophilic and hydrophobic residues. Such layout promotes β -sheet formation through intermolecular interactions. The negatively charged glutamic acids (E) and positively charged lysines (K) are on the upper side of the backbone and the hydrophobic isoleucines (I) are on the lower side. B: Light microscope image of nasal chondrocytes encapsulated in peptide hydrogel at day 1. Cells are indicated by red arrows (scale bar = 10 μ m). C: IEIK13 hydrogel supports cell growth. The chondrocytes were amplified for 2 weeks on plastic in the presence of 10% FBS supplemented with FI and were then encapsulated in hydrogel and cultivated for 3 weeks with ITS and ascorbic acid (CTRM culture medium). After 7, 14, and 21 days of culture in IEIK13 hydrogel, cells were counted. Data are presented with median as a bar ($n = 3$). * indicates statistically significant differences (* $p < 0.1$).

Gene expression analysis by reverse transcription polymerase chain reaction

Total RNA was isolated from the chondrocytes cultured in hydrogels after a 21-day culture period using the Nucleospin RNA II kit (Macherey-Nagel), according to manufacturer's instructions. Reverse transcription (RT) was performed with Primer script™ RT reagent kit (Clontech) and oligo(dT₁₂VN) primers (V represents A, G, or C, and N represents A, T, G, or C). A 30- μ L reaction was prepared by mixing 20 ng of total RNA with 6 μ L 5 \times PrimeScript Buffer, 0.5 μ L PrimeScript RT enzyme Mix I, 1.5 μ L Oligo dT primer (50 μ M), 6 μ L oligo(dT₁₂VN) primers (30 μ M), and 1 μ L RNase-free water. Reactions were carried out at 37°C for 15 min, followed by an inactivation of the enzyme at 85°C for 10 s. The cDNAs were then incubated with 1 μ L of 2 units/ μ L RNase H (Clontech) at 37°C for 30 min. For real-time polymerase chain reaction (Rt-PCR) amplification, each 16 μ L reaction contained 4 μ L of RT-reaction (diluted 1:3), 1 μ L each primer (6 μ M), and 10 μ L Fast Start Universal SYBR Green Master (Roche). Primer sequences were derived from the literature (Table I). Amplification was performed with a Rotor-gene Q cycler (Qiagen). After an initial denaturation step of 2 min at 95°C, amplification consisted of 40 cycles of 15 s at 95°C and annealing and extension at 60°C for 30 s. Results were expressed as relative values normalized with the reference gene ribosomal protein (*RPL30*) and quantified by the $\Delta\Delta$ Ct method.

Western blot analysis

After 21 days of *in vitro* culture, medium was removed from wells and IEIK13 hydrogels were re-suspended and boiled in 300 μ L 2 \times Laemmli buffer containing 3% β -mercaptoethanol. 20 μ L samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4%–15% Mini-PROTEAN® TGX™ gradient gels (Biorad). Following transfer, membranes were probed with primary antibodies, washed, and incubated with secondary antibodies (Table II). After several washes, bound antibodies were detected on X-ray films with Immun-star AP or HRP chemiluminescent substrate (Bio-Rad). When re-probed with antibodies, membranes were preliminary stripped with ReBlot Plus Strong solution (Millipore).

Histological, immunohistochemical, and immunofluorescence analysis

IEIK13 constructs cultured *in vitro* for 21 days were fixed for 24 h in formal acetic alcohol (AFA, Microm Microtech), dehydrated in a series of graded ethanol baths, and embedded in paraffin. Histological staining, immunohistochemistry (IHC), and immunofluorescence (IF) were performed on 5 μ m sections. Deparaffinized sections were stained with Goldner's Masson trichrome. For detection of proteoglycans,

TABLE I. Oligonucleotide Primers Used for the Real Time Polymerase Chain Reaction Analyses

Gene	Sequence (5' → 3')	References
<i>RPL30</i>	Forward: CCTAAGGCAGGAAGATGGTG Reverse: AGTCTGCTTGTACCCAGGA	NM_000989
<i>COL2A1</i>	Forward: TCCATGTTGCAGAAAACCTTCA Reverse: GGAAGAGTGGAGACTACTGGATTGAC	Martinez-Sanchez and Murphy ⁴²
<i>ACAN</i>	Forward: TCGAGGACAGCGAGGCC Reverse: TCGAGGGTGTAGCGTGTAGAGA	Martin <i>et al.</i> ⁴³
<i>COL9A1</i>	Forward: ACGGTTTGCCTGGAGCTAT Reverse: ACCGTCTCGGCCATTTCT	NM_001851
<i>COL10A1</i>	Forward: CAAGGCACCATTCTCCAGGAA Reverse: AAAGGGTATTTGTGGCAGCATATT	Martin <i>et al.</i> ⁴³
<i>MMP-13</i>	Forward: TCCTCTTCTTGAGCTGGACTCATT Reverse: CGCTCTGCAAACCTGGAGGTC	NM_002427
<i>COL1A1</i>	Forward: CAGCCGCTTCACCTACAGC Reverse: TTTTGTATTCAATCACTGTCTTGCC	Martin <i>et al.</i> ⁴³

sections were stained with Safranin-O in 0.1 M sodium acetate (pH 7.4) for 10 min.

IHC was performed as previously described²⁸ with the relevant antibodies (Table II). For IF, deparaffinized and rehydrated sections were incubated with 800 U/mL type IS hyaluronidase (Sigma) for 1 h at room temperature (RT) followed by cell permeabilization with 0.1% triton (Triton X-100, Sigma) for 10 min at RT. The sections were washed in PBS then incubated for 45 min at RT in 1% bovine serum albumin (BSA, Sigma) in PBS (BSA/PBS), followed by incubation with primary antibodies (Table II) diluted in BSA/PBS overnight at 4°C. The sections were washed several times with PBS and incubated with secondary antibodies (Table II) diluted in BSA/PBS for 1 h at RT. Nuclei were stained with 1× Hoechst (Fluka) in PBS for 5 min at RT. Following several PBS washes, the sections were mounted in 50% glycerol solution for observation.

Images were acquired with an ECLIPSE TI-E microscope (Nikon) coupled to a color (DS-Fi2) or a monochrome (DSQ12-ND) camera using NIS-Elements imaging software (Nikon).

Statistical analysis

Quantitative differences in cell proliferation, relative gene expression, and protein synthesis between experimental groups were analyzed using the nonparametric Mann-Whitney *U* test. The *p* values are indicated in figure legends.

RESULTS

Cell proliferation in hydrogel

We first evaluated the capacity of IEIK13 to support the proliferation of HNC. The chondrocytes were amplified for 2 weeks on plastic in the presence of 10% FBS supplemented with FI and were then encapsulated in hydrogels and

TABLE II. Primary and Secondary Antibodies

	Target Protein	Antibodies	Dilution	Source
Primary Antibodies	Type I collagen	Polyclonal anti-type I collagen	IB: 1:3000 IH: 1:2000	Novotec (Ref. 20111)
	Type II collagen	Polyclonal anti-type II collagen	IB: 1:2500 IH: 1:500	Novotec (Ref. 20211) Le Guellec <i>et al.</i> ⁴⁴
	Type II collagen	Monoclonal anti-type II collagen (Clone 6B3)	IF: 1:100	Millipore (Ref. MAB8887)
	Type IX collagen	Monoclonal anti-type IX collagen (Clone 23-5D1)	IB: 1:3000	Millipore (Ref. MAB3304)
	Type X collagen	Monoclonal anti-type X collagen (Clone X53)	IH: 1:30	eboscience (Ref. 14-9771).
	Sox9	Polyclonal anti-Sox9	IB: 1:3000 IF: 1:100	Millipore (Ref. AB5535)
	Actin	Polyclonal anti-Actin	IB: 1:2000	Sigma (Ref. A2066)
Secondary Antibodies		IgG AP-conjugated anti-rabbit	1:3000	Cell Signaling technology (Ref. 05/2016)
		IgG HRP-conjugated anti-mouse	1:3000	Cell Signaling Technology (Ref. 11/2010)
		IgG HRP-conjugated anti-mouse	Undiluted	Dako (Ref. K4002)
		IgG HRP-conjugated anti-rabbit	Undiluted	Dako (Ref. K4002)
		Alexa Fluor 546-conjugated anti-mouse Alexa Fluor 488-conjugated anti-rabbit	1:500 1:500	Invitrogen (Ref. A11030) Invitrogen (Ref. A11034)

IF, immunofluorescence; IH, immunohistochemistry; IB, immunoblotting.

cultivated for 3 weeks with ITS and ascorbic acid (CTRm culture medium). Self-assembling of IEIK13 sequences organized a 3D microenvironment in which chondrocytes appeared rounded on the day of their embedding [Fig. 1(B)]. The chondrocyte population increased during the first week of culture in hydrogel, then decreased during the second week, and remained stable afterward [Fig. 1(C)].

Gel consistency and architecture at the end of the 3D culture

After 21 days in culture, the IEIK13-HNC mixtures formed gels whose consistency and tissular organization were different depending on the culture conditions. After culture with BIT, gels appeared more homogeneously spread in the culture wells when compared to the control conditions [Fig. 2(A,B)]. In addition, BIT-treated gels could easily be recovered by a spatula and stick to it but gels formed in control conditions were consistently more liquid, making this handling not possible [Fig. 2(C)]. Observation of histological sections revealed that cells encapsulated in BIT-treated gels appeared clearly round, a morphology typical of well-differentiated chondrocytes [Fig. 2(E)]. Of note, this round morphology was not as easily observed in control gels, where chondrocytes appeared rather starry [Fig. 2(D)]. In addition, staining with Goldner's Masson trichrome evidenced abundant presence of collagen that was neo-synthesized in BIT-treated gels but not in control gels [Fig. 2(D,E)].

Gene expression analysis in the hydrogels

Gene expression analysis was first performed to quantify the relative abundance of the transcripts coding for type II and type IX collagens and aggrecan, characteristic proteins of the

cartilage matrix. After amplification on plastic, HNC were cultivated for 21 days in IEIK13 gels, in the presence or absence of BIT. The addition of BIT significantly increased the levels of expression of *COL2A1*, *COL9A1*, and *AGGRECAN* in comparison with the levels obtained in control conditions [Fig. 3(A-C)]. We also analyzed *COL1A1*, coding for type I procollagen $\alpha 1$ chain, a marker classically expressed by dedifferentiated chondrocytes. The chondrogenic BIT cocktail did not significantly stimulate *COL1A1* [Fig. 3(D)]. Finally, we measured the levels of expression of *COL10A1* coding for type X procollagen and of *MMP-13* coding for matrix metalloproteinase-13, two markers of hypertrophic chondrocytes engaged further in the pathway of chondrocyte differentiation and not wanted in hyaline cartilage (Fig. 4). We found that the expression levels of *COL10A1* and *MMP-13* were respectively higher and lower in the presence of BIT, when compared to the levels measured in control gels [Fig. 3(E,F)]. The uncoupled effect of BIT on these two markers was rather an indication that the chondrocytes did not engage hypertrophic maturation.

Characterization of the neo-synthesized matrix

In parallel cultures, we analyzed by Western blotting the proteins newly synthesized by the chondrocytes encapsulated within hydrogels. Type II and type IX collagens were not or barely detected in control conditions, whereas BIT treatment clearly stimulated their synthesis [Fig. 5(A-C)]. Type I collagen was produced to equivalent levels in the two culture conditions [Fig. 5(A,D)]. Consequently, the type II/type I collagen ratio measured by densitometric analysis of the Western blots and taken as an index of chondrocyte differentiation, was higher in BIT-treated gels [Fig. 5(E)]. More precisely and very

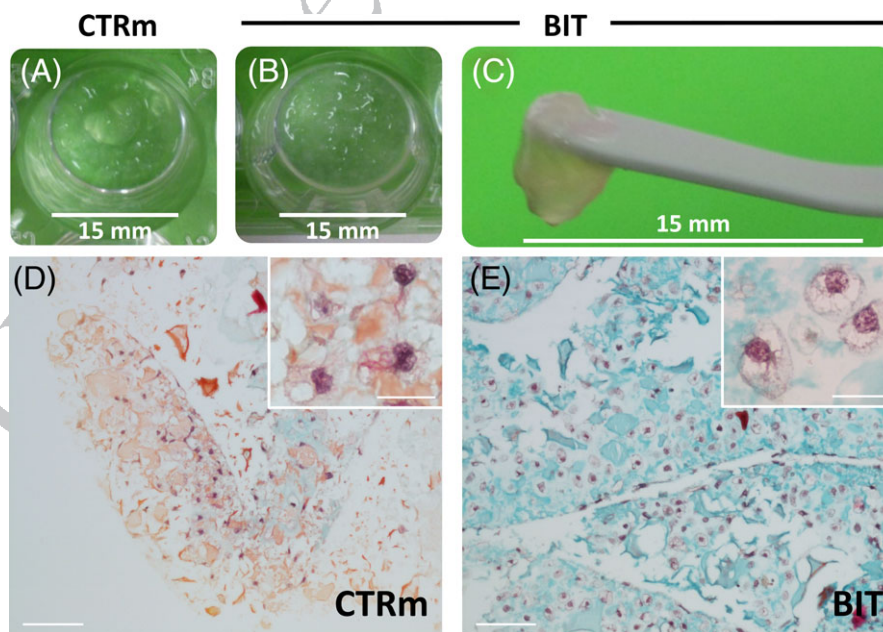


FIGURE 2. A–C: Macroscopic aspect of chondrocyte-seeded IEIK13 hydrogels at the end of the *in vitro* culture. Top views of (A) CTRm-treated and (B) BIT-treated constructs in culture wells. C: Side view of a BIT-treated construct sticking to a spatula. D, E: Goldner's Masson trichrome staining of cells encapsulated in IEIK13 hydrogel after 2 weeks of amplification with FI and 3 weeks of redifferentiation in peptide hydrogel in the presence of (D) CTRm or (E) BIT. Nuclei are colored in purple, cytoplasm in red, and collagens in green (scale bars = 100 μm , insert scale bars = 20 μm).

Chondrogenic genes

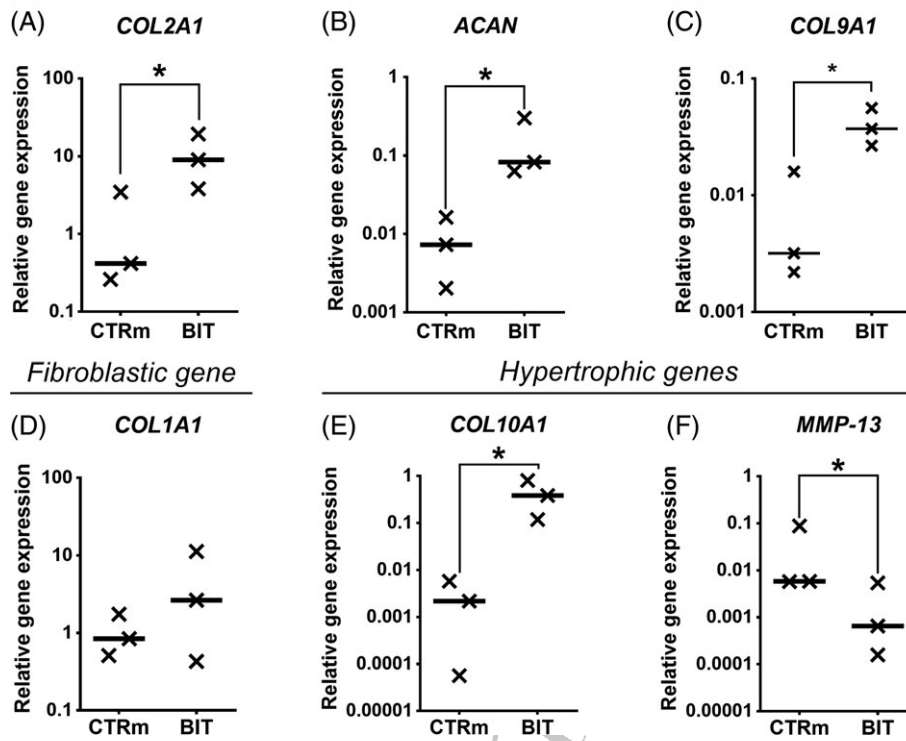


FIGURE 3. Gene expression characterization of HNC cultivated for 21 days in IEIK13 hydrogel. The chondrocytes were amplified for 2 weeks on plastic in the presence of 10% FBS supplemented with FI and were then encapsulated in hydrogel and cultivated for 3 weeks in control medium (CTRm) or in chondrogenic medium containing BIT. Data are presented as box plots with median as a bar ($n = 3$). * indicates statistically significant differences (* $p < 0.05$).

interestingly, type II collagen was present as mature forms but also abundantly as unprocessed (proforms) in BIT conditions, indicating that cells were still actively producing type II collagen after 21 days of culture. In parallel, the presence of type I collagen was restricted as mature and processing intermediate chains, but not as total, unprocessed chains that were only visible in control conditions [Fig. 5(A)]. This was a sign that HNC stopped their production of type I collagen, when treated with BIT.

To have a more general view of the distribution of the ECM produced by HNC in IEIK13 hydrogels, we also performed immunohistochemistry for type I, type II, and type X collagen and we used Safranin-O staining to reveal the presence of glycosaminoglycans (GAGs) of sulfated proteoglycans (Fig. 6). Our observations clearly showed that BIT treatment resulted in superior accumulation and homogenous distribution of type II collagen and GAGs in IEIK13 hydrogels, compared to control conditions. In contrast, immunostaining for type I collagen appeared similarly weak in control and BIT conditions. Interestingly, in BIT-treated gels, we found type II collagen in cell cytoplasm, whereas type I collagen was detected in the extracellular space but not in cell cytoplasm, suggesting that HNC were no longer actively synthesizing type I collagen. This observation is in full concordance with our Western-blot data indicating a stop in production of type I collagen. Last, immunostaining for type X collagen appeared extremely weak in hydrogels treated or not with

BIT. Of note, in BIT-treated gels, a few cells showed intracellular staining for type X collagen but no signs of cellular hypertrophy.

Sox9 translocation as a sign of good chondrogenic activity in BIT-treated hydrogels

To provide further evidence that HNC encapsulated in IEIK13 hydrogel and treated with BIT corresponded to functionally active chondrocytes, we looked for Sox9 production and cellular localization. Sox9 is a major factor in developing and adult cartilage. It fulfills many important functions during chondrogenesis including transcriptional activation of many genes coding cartilage-specific structural components such as types II and IX collagens and aggrecan³⁰ studied here. Our Western blotting analysis confirmed that Sox9 was expressed by HNC, with no difference in the level of synthesis between control and BIT conditions [Fig. 7(A,B)]. We then looked more carefully at the cellular distribution of Sox9, depending on the culture conditions. We first observed by immunofluorescence that type II collagen was abundantly synthesized and deposited in the extracellular space by BIT-treated chondrocytes, but was almost undetectable in gels cultivated in control conditions [Fig. 7(C)]. Close examination of Sox9 by immunofluorescence revealed an intense nuclear staining in BIT-treated HNC, whereas the staining was cytoplasmic in HNC cultivated in control conditions [Fig. 7(C)]. These observations indicated that the enhanced deposition

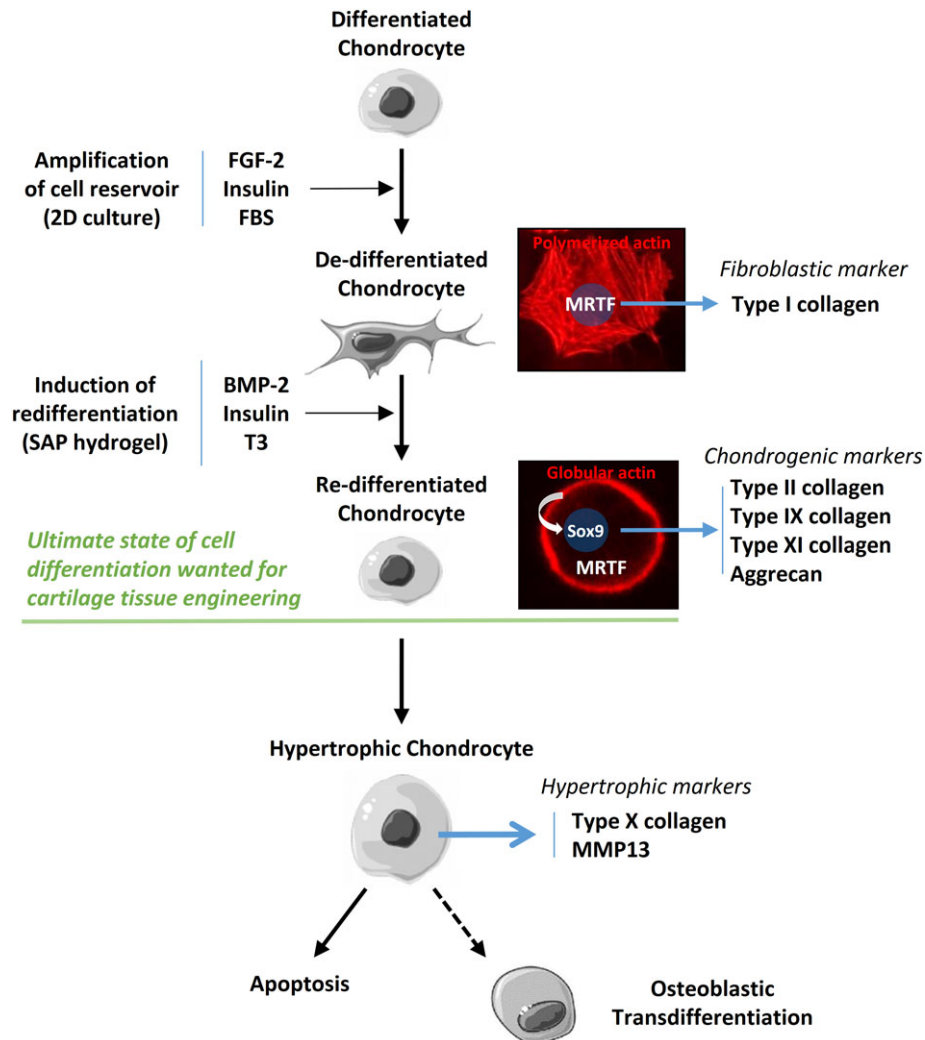


FIGURE 4. Diagram recapitulating the evolution of the chondrocyte phenotype in 2D and 3D cultures. These culture conditions are generally used to amplify the cell reservoir and to reconstruct cartilage in the context of cartilage tissue engineering. When seeded in monolayer (2D culture), chondrocytes spread and show increased actin polymerization and high proportion of myocardin-related transcription factor-a (MRTF-a) in the nucleus. MRTF-a is an activator of type I collagen, unwanted in cartilage. When seeded in hydrogel such as SAP (3D culture), the chondrocytes become round. With this round shape, depolymerization of filamentous (f-) actin into globular (g-) actin results in MRTF-a binding to g-actin and localization of MRTF-a in the cytoplasm, thus leading to a decrease in type I collagen expression.³² Concomitantly, loss of polymerized actin corresponds to loss of RhoA protein and its activity, leading to enhancement of Sox9 function such as type II collagen activation.³¹ This cellular activity corresponds to a status of well-differentiated chondrocyte required to engineer hyaline cartilage. It should also be kept in mind that chondrocytes have the ability to engage forward to hypertrophic maturation during endochondral ossification. In this developmental process, most hypertrophic chondrocytes undergo apoptosis but some hypertrophic chondrocytes can transdifferentiate into osteoblasts, as reported recently.⁴¹ This flexibility of the chondrocyte phenotype implies to verify that chondrocytes do not express markers characteristic of dedifferentiated or hypertrophic chondrocytes when they are used in the protocols of cell therapy and tissue engineering of cartilage.

of type II collagen around the BIT-treated chondrocytes was associated with selective localization of Sox9 in the nucleus. Thus, stimulation of the chondrocytes with BIT induced nuclear translocation of Sox9 and this is in concordance with the transcriptional function of Sox9 expected for active chondrocytes.

DISCUSSION

The biomaterials with various internal structures provide different microenvironments for the chondrocytes that influence their function. Hydrogels are particularly convenient since they induce cell rounding and round morphology associated with

depolymerized actin is essential for correct chondrogenic expression.³¹ On the contrary, in a 2D environment such as a phase of cellular expansion on plastic, chondrocytes adopt a fibroblastic shape associated with polymerized actin and this results in chondrocyte dedifferentiation.³² In this study, we hypothesized that an SAP IEIK13 hydrogel would provide an appropriate 3D environment for encapsulation of chondrocytes. Because our aim was to develop a cartilage gel for nasal cartilage engineering applications, we used here HNC. We also used cocktails of specific factors to expand the cells on plastic (cocktail F1) and to allow their redifferentiation in IEIK13 hydrogel (cocktail BIT). Our approach to develop a cartilage-like hydrogel

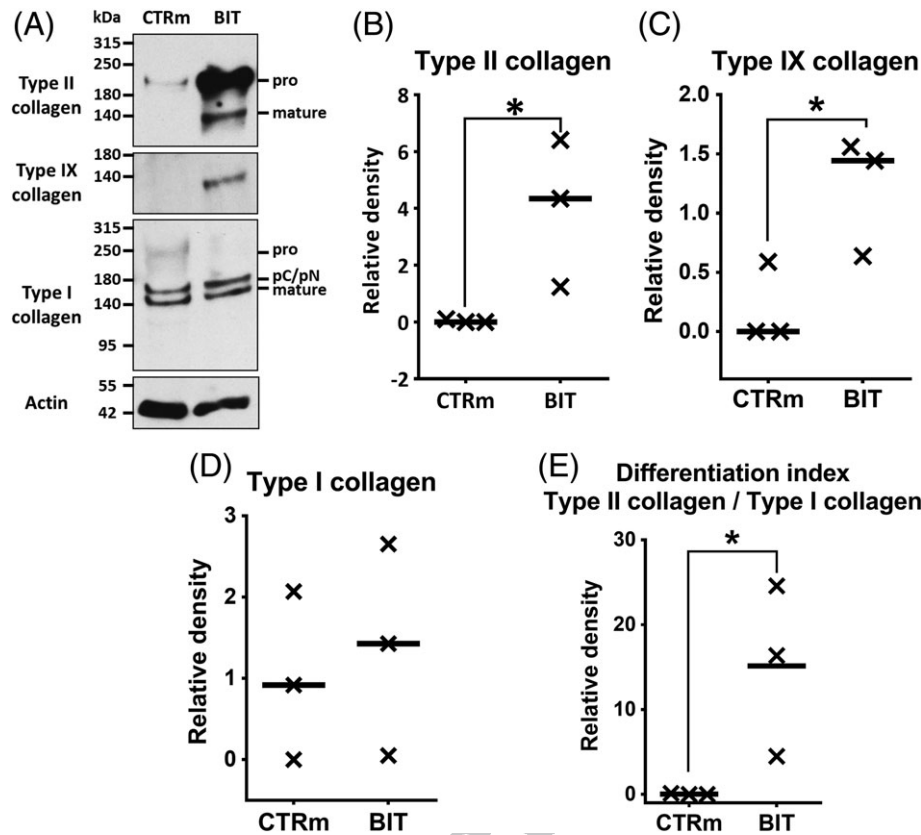


FIGURE 5. Treatment with BIT favors cartilage-characteristic matrix reconstruction in IEIK13 hydrogel. The chondrocytes were amplified for 2 weeks on plastic in the presence of 10% FBS supplemented with FI and were then encapsulated in hydrogel and cultivated for 3 weeks in control medium (CTRm) or in chondrogenic medium containing BIT. A: Representative WB analysis of types I, II, and IX collagens. The positions of mature collagen chains (mature), unprocessed (pro), or processing intermediates of the procollagen containing the carboxypropeptide (pC) or the aminopropeptide (pN) are indicated. Actin bands are shown as control for equivalent loading of the extracts. B-E: Quantification of type II, type IX, type I collagen, and type II collagen/type I collagen through the densitometric analysis of Western blots data. Total collagens (mature, unprocessed, or processing intermediates) were normalized to actin. The values given in the dot plots represent single data point with mean ($n = 3$). * indicates statistically significant differences ($*p < 0.05$).

combining HNC, soluble factors, and the self-assembling IEIK13 peptide is summarized in Figure 4. Ultimately, this cartilage gel could be used in combination with a porous polymeric nasal implant to ensure its biotolerance.

Above all, our cell proliferation study indicates that the peptide hydrogel matrix appears favorable for chondrocyte division. The slowdown in growth rate observed after the first week of culture likely results from the privation of growth factors in the culture medium chosen to study more specifically effect of the peptide on cell behavior. Then, when BIT or CTRm was supplemented to the chondrocytes cultivated in gel, our histological observations revealed that the structure of the chondrocyte-IEIK13 constructs was rather organized in blocks of gel containing cells. This contrasts with the results obtained with other SAPs such as KLD12 and RADA16-I which seemed to present a more homogeneous texture when combined to chondrocytes.^{21,25} This could be explained by the difference in composition and/or number of amino acids rather than in charge arrangement between the peptide sequences, since IEIK13, KLD12, and RADA16-I are all modulus I SAPs.¹³ Besides, we used here IEIK13 at a concentration of 1%, whereas KLD12 and

RADA16-I were used previously at the concentrations of 0.3%²⁵ or 0.5%.^{21,33} It is therefore possible that the concentration of SAP hydrogel also influences homogeneity in gelation and consequently the final structure of the gel.

After encapsulation, chondrocytes showed a round morphology within the peptide gel, similar to that usually observed in agarose culture.³⁴⁻³⁶ Thus, IEIK13 peptide allows intimate cell-scaffold interactions favorable for chondrocytes. More precisely, this round morphology was well maintained only in the presence of BIT, suggesting that the combination of the peptide and BIT is necessary to sustain the chondrocyte phenotype.

The status of the well-differentiated chondrocyte phenotype in the presence of BIT was further confirmed at the molecular level. Exposure to BIT was clearly efficient to stimulate chondrogenic genes without enhancing significantly expression of *COL1A1*. In addition, the opposite effect of BIT observed on the expressions of *COL10A1* and *MMP-13*, two genes co-expressed in hypertrophic chondrocytes³⁷ suggests that cells did not engage really toward hypertrophic maturation. In support of this view, only a very few cells stained positive for type X collagen and these cells did

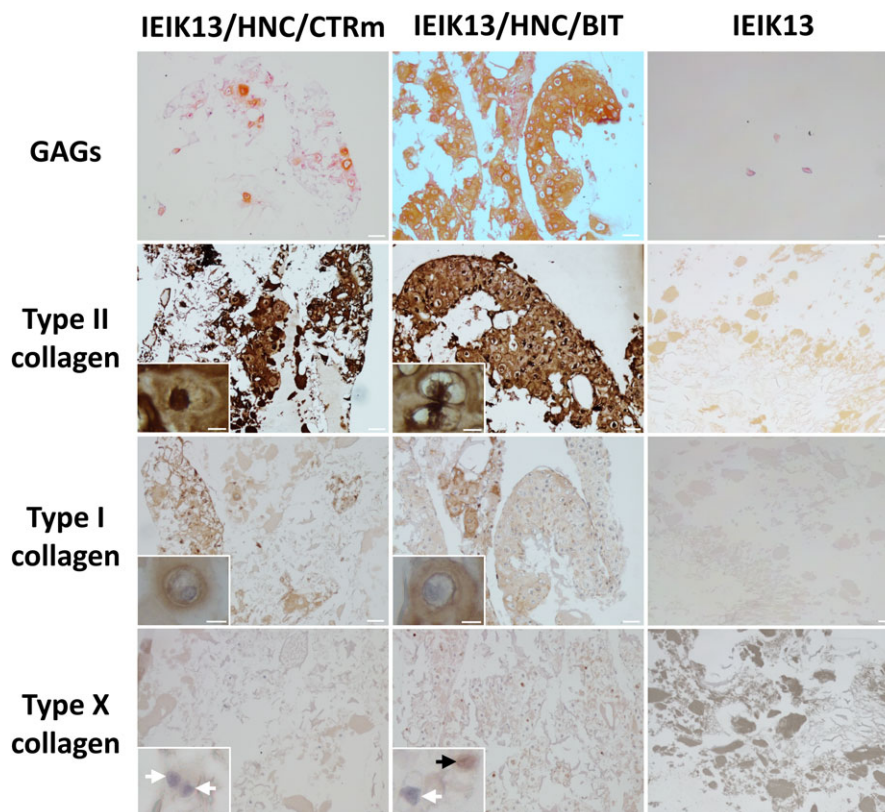


FIGURE 6. BIT treatment enhances ECM production in IeIK13 hydrogel. HNC were first amplified for 2 weeks on plastic in the presence of 10% FBS supplemented with FI, then encapsulated in hydrogel and cultivated for 3 weeks in control medium (CTRm) or in chondrogenic medium containing BIT, as indicated. Representative pictures show parallel sections of constructs stained for types I, II, and X collagens and GAGs, as indicated. As control for nonspecific background staining, acellular IeIK13 gel was also stained for types I, II, and X collagens and GAGs (right panels). In the middle panels, inserts show intracellular localization of type II collagen in gel treated with CTRm or BIT, intracellular localization of type I collagen in gel treated with CTRm but absence of type I collagen in the cell cytoplasm in gel treated with BIT. In the lower panels, inserts show cells that are positively or negatively stained for type X collagen. Negative cells appear blue (white arrow) and positive cells appear brown (black arrow) (scale bars = 50 μm , insert scale bars = 10 μm).

not show increase in cytoplasmic volume. Moreover, we paid particular attention to the nature of the newly synthesized matrix. Our Western blots data showed that BIT-treated chondrocytes produced type II and type IX collagens, two main components of the collagen fibrils present in hyaline type of cartilage.³⁸ It should be specified that the chondrocytes were most certainly partially dedifferentiated at the time they were mixed with the IeIK13 peptide, after amplification on plastic. Indeed, we have confirmed in a previous study that *COL2A1* expression decreases, whereas *COL1A1* expression increases when HNC are amplified on plastic,²⁷ as expected for chondrocytes in monolayer. Thus, the chondrocytes were active in type I collagen production at the start of their culture in IeIK13 gel and this production stopped in the presence of BIT, as confirmed by the absence of precursor forms of type I collagen in our Western blots and of intracellular type I collagen staining in our histological sections. This stop in type I collagen production is particularly relevant since it indicates that the combination of IeIK13 and BIT favors the production of hyaline type of cartilage as nasal cartilage, not fibrocartilage. This is well supported by the differentiation index (type II collagen/type I collagen ratio) and our histological staining

analyses showing abundant presence of type II collagen and GAGs, whereas type I collagen was restricted to limited areas of the cartilage gel. These results are also in line with the inhibitory role of the cocktail BIT on type I collagen expression shown in previous studies.^{26,39,40} Taken together, our results indicate BIT induced redifferentiation of chondrocytes in IeIK13 hydrogel, whereas chondrocytes continued to dedifferentiate in the gel, in the presence of CTRm. Importantly, the abundant expression of type II procollagen forms and the nuclear translocation of Sox9 occurring in cells cultivated in IeIK13 hydrogel with BIT attest that cells were active at the end of the culture period. Thus, our study demonstrates that IeIK13 SAP is a material compatible with proper chondrocyte physiology.

In summary, this study represents the first report on the use of IeIK13 SAP. Here, we demonstrated that IeIK13 is a suitable scaffold to allow expression of differentiated chondrocytes and synthesis of cartilage matrix in a 3D cell culture. Our results show that the combination of IeIK13 SAP, HNC, and BIT forms a cartilage gel that could be useful for tissue engineering of nasal cartilage. Our ongoing studies are aimed to investigate the capacity of this cartilage gel to penetrate and coat diverse porous materials with the aim to

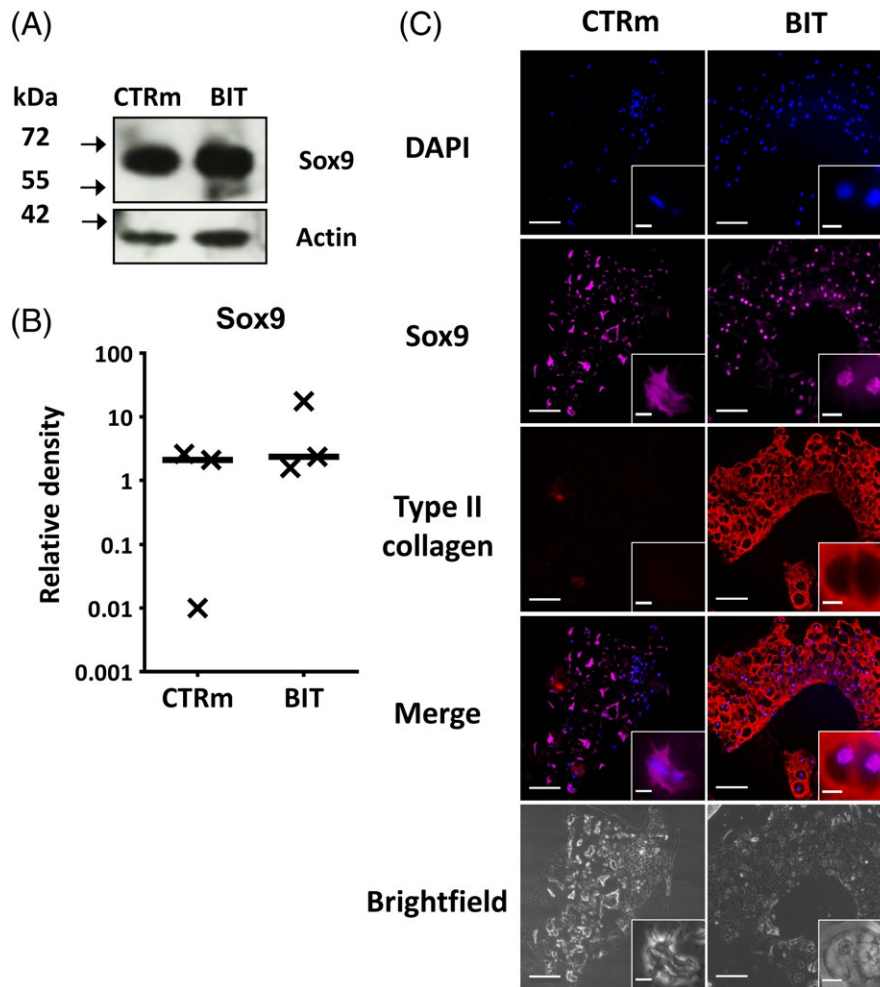


FIGURE 7. BIT treatment provokes nuclear translocation of Sox9. The chondrocytes were amplified for 2 weeks on plastic in the presence of 10% FBS supplemented with FI and were then encapsulated in IEIK13 hydrogel and cultivated for 3 weeks in control medium (CTRm) or in chondrogenic medium containing BIT, as indicated. A: Western blot analysis of Sox9 synthesis at the end of the culture period. Actin bands are shown as control for equivalent loading of the extracts. B: Quantification of Sox9 through the densitometric analysis of Western blot data. Sox9 was normalized to actin. The values given in the dot plots represent single data point with mean ($n = 3$). * indicates statistically significant differences ($*p < 0.1$). C: Immunofluorescence staining of HNC embedded in hydrogels. Cells were double-stained for Sox9 with the Alexa Fluor 488-conjugated secondary antibody (purple) and for type II collagen with the Alexa Fluor 546-conjugated secondary antibody (red). Nuclei were stained with Hoechst dye (blue) (scale bars = 100 μm, insert scale bars = 10 μm).

create nasal cartilage implants with high biocompatibility. In this context, *in vivo* studies will also be performed to ensure that the IEIK13 sequence does not elicit an immunogenic response in host animal model.

ACKNOWLEDGMENTS

The authors thank the technical facilities of SFR Biosciences Gerland-Lyon (UMS3444/US8) for the quantitative PCR analyses. This research was funded by CNRS, Lyon 1 University and by Fondation de l'Avenir. AD was supported by a PhD fellowship from the Région Rhône-Alpes (ARC2).

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