

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

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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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18 Abstract: Nasal reconstruction remains a challenge for every 19 reconstructive surgeon. Alloplastic implants are proposed to repair 20 nasal cartilaginous defects but they are often associated with high 21 rates of extrusion and infection and poor biocompatibility. In this context, a porous polymeric scaffold filled with an autologous 22 cartilage gel would be advantageous. In this study, we evaluated 23 the capacity of IEIK13 self-assembling peptide (SAP) to serve as 24 support to form such cartilage gel. Human nasal chondrocytes 25 (HNC) were first amplified with FGF-2 and insulin, and then redif-26 ferentiated in IEIK13 with BMP-2, insulin, and T3 (BIT). Our results 27 demonstrate that IEIK13 fosters HNC growth and survival. HNC 28 phenotype was assessed by RT-PCR analysis and neo-synthesized 29 extracellular matrix was characterized by western blotting and 30

immunohistochemistry analysis. BIT-treated cells embedded in IEIK13 displayed round morphology and expressed cartilagespecific 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

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INTRODUCTION

40 Nose is the primary portal of entry for the respiratory system 41 in human. It acts as an air conditioner and a defender of the 42 lower respiratory tract by filtering, humidifying, and heating 43 the inhaled air. It also serves as the major sensory organ for 44 smell. Besides these physiological functions, the nose plays a 45 critical role in harmony and radiance of the face because of its 46 central position. The nasal cavity is divided into two by a key 47 cartilage structure, the nasal septum. In case of defect, nasal 48 cartilage is not able to regenerate because of its lack of vascu-49 lature and isolation from systemic regulation, and thus nasal 50 reconstruction remains a challenge. Autologous or homologous 51

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 infection²⁻⁴ 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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(SAPs) have recently emerged as a class of next-generation 1 2 hydrogels for tissue engineering applications. These synthetic 3 materials consist of short peptide sequences that are able to self-assemble and form a tridimensional network that mimics 4 5 extracellular matrix (ECM) environment when peptide concentration and physical conditions are adequate.⁷⁻¹² The peptide 6 7 sequence is composed of alternating hydrophilic and hydro-8 phobic amino acids that form stable β -sheet structures in 9 deionized water and self-assemble into interweaving nanofi-10 bers when exposed to an electrolyte solution such as cell culture medium.⁷⁻¹² In contrast to other natural material-based 11 hydrogels such as collagen, fibrin, alginate, or hyaluronan, 12 advantages of such synthetic material include low risks of bio-13 14 logical pathogen transmission and homogeneous batches in production.^{13,14} Moreover, studies have shown that SAPs do 15 not elicit immunogenic or inflammatory response in vitro¹⁵ or 16 in vivo.^{10,16} 17

18 In the specific context of cartilage repair, SAPs are inter-19 esting materials since the self-assembling process results in 20 highly hydrated material (more than 90% water content)¹⁷ and native cartilage is highly hydrated (80% water content),¹⁸ 21 22 a feature offering the tissue its "shock absorber" capabilities. SAPs also offer the advantage of being injectable into nonuni-23 form defects,¹⁹ thanks to their malleability and it is highly 24 25 conceivable that they can be used to infiltrate and coat porous 26 scaffolds intended to serve as implants. Moreover, SAPs have 27 been shown to sustain or allow recovery of the chondrogenic phenotype with expression of cartilage-specific matrix pro-28 teins when they are combined with rabbit,²⁰ bovine,²¹⁻²⁴ and 29 human²⁵ chondrocytes. Therefore, SAPs appear as promising 30 31 supports to form cartilage gel. Following this view, the aim of 32 this work was to evaluate if a new SAP sequence, referred as 33 IEIK13, could provide a suitable three-dimensional environ-34 ment for chondrogenic expression of human nasal chondro-35 cytes (HNC). We used cocktails of specific factors to expand 36 the cells on plastic (fibroblast growth factor (FGF)-2 and insu-37 lin, designated FI) and to allow their redifferentiation in 38 IEIK13 hydrogel (bone morphogenetic protein (BMP)-2, insu-39 lin, and triiodothyronine T3, designated BIT). This sequential 40addition of factors has been proven efficient to amplify human 41 chondrocytes isolated from diverse anatomical sites (including nose) and to induce their redifferentiation in collagen²⁶ or 42 agarose²⁷ gels and in collagen sponges.^{28,29} 43

45 MATERIALS AND METHODS

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46 Cell extraction and amplification

47 Primary cultures of HNC were prepared from healthy septal 48 cartilages of 3 donors (age range: 21-45) undergoing rhino-49 plasty. Nasal cartilage samples were collected upon written 50 informed consent of donors and complied with local ethics 51 guidelines, national and European Union legislation regard-52 ing human sample collection, manipulation, and personal 53 data protection (Ethics Committee for research with human 54 samples, CODECOH: DC-2014-2325). For chondrocyte extrac-55 tion, septal cartilages were washed with phosphate-buffered 56 saline (PBS) (Sigma-Aldrich) supplemented with 50 µg/mL 57 streptomycin (Panpharma) then cut in small slices and 58 digested overnight at 37°C with 0.5 mg/mL bacterial collagenase A (Roche Applied Science) in culture medium 59 composed of Dulbecco's modified Eagle medium/Ham's F12 60 (DMEM-F12) (Gibco/Life technologies). Chondrocytes were 61 then seeded on culture dishes at a density of 1.5×10^4 62 cells/cm² in culture medium supplemented with 50 μ g/mL 63 streptomycin and 10% fetal bovine serum (FBS) (Gibco-invi-64 trogen). At this stage, cells were designated P0. Forty-eight 65 hours after seeding, the medium was refreshed and further 66 supplemented with 5 ng/mL fibroblast growth factor (FGF)-67 2 (R&D Systems) and 5 µg/mL insulin (Umuline Rapide, 68 Lilly). The combination of FGF-2 and insulin was referred as 69 FI. FI-supplemented complete medium was replaced three 70 times a week. The cells were cultivated for 2 weeks and then 71 trypsinized. 72

Cell culture in IEIK13 self-assembling peptide hydrogel

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IEIK13 peptide solution was provided by 3-D Matrix, Inc. 75 (Needham, MA). Peptide sequence is Ac-IEIKIEIKIEIKI-NH2 76 [Fig. 1(A)]. Peptide powder was synthesized by CPC (Chinese 77 Peptide Company, Hangzhou, China) and was dissolved in 78 10% wt/vol sucrose solution at a peptide concentration of 79 1.5% (wt/vol). Passaged P0 chondrocytes were resuspended 80 in a volume of 10% wt/vol sucrose solution then mixed with 81 a volume of the 1.5% peptide solution so that the final pep-82 tide solution was 1% (wt/vol) and the cell density was 83 2×10^{6} cells/mL. The cell-peptide solution was then care-84 fully homogenized by reverse pipetting and 100 µL of gel 85 was poured in wells of a 48-well culture plate. One milliliter 86 of DMEM-F12 medium was then carefully added to each well 87 and the resulting mixed solutions were incubated at 37°C for 1 h to allow self-assembling. Finally, 500 µL of culture 89 medium was substituted by new medium so that the com-90 plete medium contained 1× ITS (insulin, transferrin, and 91 selenium, Gibco/Life technologies), 50 µg/mL 2-phospho-L-92 ascorbic acid (trisodium salt, Fluka) supplemented or not 93 with 200 ng/mL of recombinant human bone morphogenetic 94 protein (BMP)-2 (Dibotermine- α , drug form of BMP-2 con-95 tained in InductOs kit, Wyeth), 5 µg/mL insulin (Umulin, 96 Lilly), and 100 nM T3 (triiodothyronine, Sigma). The combi-97 nation of BMP-2, insulin, and T3 was referred as BIT. The 98 culture medium without BIT was designated as control 99 medium (CTRm). The medium was replaced by half every 100 2 days over a culture period of 21 days. 101

Evaluation of cell growth

After 7, 14, and 21 days of culture in IEIK13 hydrogel in 104 the presence of CTRm, 1 mL PBS was added to the wells 105 and cells were mechanically dissociated from hydrogel by 106 several reverse-pipetting then centrifuged. Following PBS 107 removal, cell-matrix interactions were further disrupted 108 with trypsin and cells were stained with Trypan blue and 109 counted with a Cellometer (Nexcelom). For each time point, 110 the rate of growth was calculated over the entire culture 111 period using the following formula: growth rate = $(\ln(T/S))/$ 112 $\ln(2)/D$, where S is the number of cells initially seeded 113 (in our case 200,000 cells for a 100 μ L gel), T is the num-114 ber of cells obtained after trypsinization, and D is the num-115 ber of days in culture. 116



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 µm). 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 scriptTM RT reagent kit (Clontech) and $oligo(dT_{12}VN)$ primers (V represents A, G, or C, and N represents A, T, G, or C). A 30-µL reaction was prepared by mixing 20 ng of total RNA with 6 μ L 5× 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 µM), and 1 µL 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 µL of 2 units/µL RNase H (Clontech) at 37°C for 30 min. For real-time polymerase chain reaction (Rt-PCR) amplification, each 16 µL reaction contained 4 µL of RT-reaction (diluted 1:3), 1 µL each primer (6 µM), and 10 µL 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 μL 2× Laemmli buffer containing 3% β-mercaptoetha-nol. 20 µL 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 sub-strate (Bio-Rad). When reprobed with antibodies, mem-branes were preliminary stripped with ReBlot Plus Strong solution (Millipore).

Histological, immunohistochemical, and immunofluorescence analysis

IEIK13 constructs cultured *in vitro* for 21 days were fixed 110 for 24 h in formol acetic alcohol (AFA, Microm Microtech), 111 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, 116

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

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

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

	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	⁷ 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)
\sim	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)
econdary 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	1:500	Invitrogen (Ref. A11030)
		Alexa Fluor 488-conjugated anti-rabbit	1:500	Invitrogen (Ref. A11034)

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

DUFOUR ET AL. cultivated for 3 weeks with ITS and ascorbic acid (CTRm cul-ture medium). Self-assembling of IEIK13 sequences orga-nized 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 α 1 chain, a marker classically expressed by ded-ifferentiated 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 fur-ther 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 respec-tively higher and lower in the presence of BIT, when com-pared 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





(D) CTRm or (E) BIT. Nuclei are colored in purple, cytoplasms in red, and collagens in green (scale bars = 100 µm, insert scale bars = 20 µm).



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 condi-tions, 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 inter-mediate 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 pres-ence of glycosaminoglycans (GAGs) of sulfated proteoglycans (Fig. 6). Our observations clearly showed that BIT treatment resulted in superior accumulation and homogenous distribu-tion of type II collagen and GAGs in IEIK13 hydrogels, com-pared 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 func-tionally 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 dur-ing 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 synthe-sis 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 cyto-plasmic in HNC cultivated in control conditions [Fig. 7(C)]. These observations indicated that the enhanced deposition

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amplify the cell reservoir and to reconstruct cartilage in the context of cartilage tissue engineering. When seeded in monolayer (2D culture), chon-37 95 drocytes spread and show increased actin polymerization and high proportion of myocardin-related transcription factor-a (MRTF-a) in the nucleus. 38 MRTF-a is an activator of type I collagen, unwanted in cartilage. When seeded in hydrogel such as SAP (3D culture), the chondrocytes become 96 round. With this round shape, depolymerization of filamentous (f-) actin into globular (g-) actin results in MRTF-a binding to g-actin and localization 39 97 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 40 98 41 99 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 100 42 undergo apoptosis but some hypertrophic chondrocytes can transdifferentiate into osteoblasts, as reported recently.⁴¹ This flexibility of the chon-101 43 drocyte phenotype implies to verify that chondrocytes do not express markers characteristic of dedifferentiated or hypertrophic chondrocytes when 44 102 they are used in the protocols of cell therapy and tissue engineering of cartilage. 103

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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.

54 **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 105 expression.³¹ On the contrary, in a 2D environment such as a 106 phase of cellular expansion on plastic, chondrocytes adopt a 107 fibroblastic shape associated with polymerized actin and this 108 results in chondrocyte dedifferentiation.³² In this study, we 109 hypothesized that an SAP IEIK13 hydrogel would provide an 110 appropriate 3D environment for encapsulation of chondrocytes. 111 Because our aim was to develop a cartilage gel for nasal carti-112 lage engineering applications, we used here HNC. We also used 113 114 cocktails of specific factors to expand the cells on plastic (cocktail FI) and to allow their redifferentiation in IEIK13 hydrogel 115 (cocktail BIT). Our approach to develop a cartilage-like hydrogel 116

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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.

41 Above all, our cell proliferation study indicates that the 42 peptide hydrogel matrix appears favorable for chondrocyte division. The slowdown in growth rate observed after the 43 44 first week of culture likely results from the privation of 45 growth factors in the culture medium chosen to study more 46 specifically effect of the peptide on cell behavior. Then, when 47 BIT or CTRm was supplemented to the chondrocytes culti-48 vated in gel, our histological observations revealed that the 49 structure of the chondrocyte-IEIK13 constructs was rather 50 organized in blocks of gel containing cells. This contrasts 51 with the results obtained with other SAPs such as KLD12 52 and RADA16-I which seemed to present a more homogeneous texture when combined to chondrocytes.21,25 This 53 54 could be explained by the difference in composition and/or 55 number of amino acids rather than in charge arrangement between the peptide sequences, since IEIK13, KLD12, and 56 RADA16-I are all modulus I SAPs.¹³ Besides, we used here 57 58 IEIK13 at a concentration of 1%, whereas KLD12 and RADA16-I were used previously at the concentrations of 95 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. 98

99 After encapsulation, chondrocytes showed a round morphology within the peptide gel, similar to that usually 100 observed in agarose culture.³⁴⁻³⁶ Thus, IEIK13 peptide 101 allows intimate cell-scaffold interactions favorable for 102 chondrocytes. More precisely, this round morphology was 103 well maintained only in the presence of BIT, suggesting 104 that the combination of the peptide and BIT is necessary 105 to sustain the chondrocyte phenotype. 106

The status of the well-differentiated chondrocyte phe-107 notype in the presence of BIT was further confirmed at the 108 molecular level. Exposure to BIT was clearly efficient to 109 stimulate chondrogenic genes without enhancing signifi-110 cantly expression of COL1A1. In addition, the opposite effect 111 of BIT observed on the expressions of COL10A1 and MMP-112 13, two genes co-expressed in hypertrophic chondrocytes³⁷ 113 suggests that cells did not engage really toward hypertro-114 phic maturation. In support of this view, only a very few 115 cells stained positive for type X collagen and these cells did 116

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Findence V. Bit relating the inflatest Eckly production in Encry hydroger. Index were instant aniphined for 2 weeks on plaste in the presence of 10/x Hos 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 or 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).

36 not show increase in cytoplasmic volume. Moreover, we 37 paid particular attention to the nature of the newly synthe-38 sized matrix. Our Western blots data showed that BIT-39 treated chondrocytes produced type II and type IX collagens, two main components of the collagen fibrils present 40 in hyaline type of cartilage.³⁸ It should be specified that the 41 chondrocytes were most certainly partially dedifferentiated 42 43 at the time they were mixed with the IEIK13 peptide, after 44 amplification on plastic. Indeed, we have confirmed in a 45 previous study that COL2A1 expression decreases, whereas COL1A1 expression increases when HNC are amplified on 46 plastic,²⁷ as expected for chondrocytes in monolayer. Thus, 47 48 the chondrocytes were active in type I collagen production 49 at the start of their culture in IEIK13 gel and this produc-50 tion stopped in the presence of BIT, as confirmed by the 51 absence of precursor forms of type I collagen in our West-52 ern blots and of intracellular type I collagen staining in our 53 histological sections. This stop in type I collagen production 54 is particularly relevant since it indicates that the combina-55 tion of IEIK13 and BIT favors the production of hyaline 56 type of cartilage as nasal cartilage, not fibrocartilage. This 57 is well supported by the differentiation index (type II colla-58 gen/type I collagen ratio) and our histological staining

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94 analyses showing abundant presence of type II collagen and GAGs, whereas type I collagen was restricted to limited 95 areas of the cartilage gel. These results are also in line with 96 the inhibitory role of the cocktail BIT on type I collagen 97 expression shown in previous studies.^{26,39,40} Taken together, 98 our results indicate BIT induced redifferentiation of chondro-99 cytes in IEIK13 hydrogel, whereas chondrocytes continued to 100 101 dedifferentiate in the gel, in the presence of CTRm. Importantly, the abundant expression of type II procollagen forms 102 and the nuclear translocation of Sox9 occurring in cells culti-103 vated in IEIK13 hydrogel with BIT attest that cells were active 104 at the end of the culture period. Thus, our study demonstrates 105 that IEIK13 SAP is a material compatible with proper chon-106 107 drocyte physiology.

In summary, this study represents the first report on the 108 use of IEIK13 SAP. Here, we demonstrated that IEIK13 is a 109 suitable scaffold to allow expression of differentiated chon-110 drocytes and synthesis of cartilage matrix in a 3D cell cul-111 ture. Our results show that the combination of IEIK13 SAP, 112 HNC, and BIT forms a cartilage gel that could be useful for 113 tissue engineering of nasal cartilage. Our ongoing studies are 114 aimed to investigate the capacity of this cartilage gel to pene-115 trate and coat diverse porous materials with the aim to 116

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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.

(blue) (scale bars = 100 μ m, insert scale bars = 10 μ m).

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