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CULTIVATION OF AURICULAR CHONDROCYTES IN POLY(ETHYLENE GLYCOL)/POLY(ε-CAPROLACTONE) HYDROGEL FOR TRACHEAL CARTILAGE TISSUE ENGINEERING IN A RABBIT MODEL

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Abstract

Tissue engineering has the potential to overcome the limitations of tracheal reconstruction. To tissue-engineer a tracheal cartilage, auricular chondrocytes were encapsulated in a photocurable poly(ethylene glycol)/poly(ε-caprolactone) (PEG/PCL) hydrogel. Chondrogenic genes, including *Sox9*, *Acan* and *Col2a1*, were up-regulated in auricular chondrocytes after 2 weeks of *in vitro* cultivation in the PEG/PCL hydrogel. Co-cultivation of 70 % auricular chondrocytes and 30 % bone marrow mesenchymal stem cells (BMSCs) accelerated the chondrogenic genes' expression in the PEG/PCL hydrogel. Cartilaginous matrix markers, including proteoglycans and collagen type II, were detected in the chondrocytes-encapsulated PEG/PCL hydrogel after 4 weeks of *in vitro* cultivation. The higher expression level of cartilaginous matrix markers was observed in the PEG/PCL hydrogel with co-cultivation of 70 % chondrocytes and 30 % BMSCs. After 4 weeks of ectopic cultivation in rabbits, the cylindrical PEG/PCL structure was sustained with the use of a luminal silicon stent. However, without the stent, the construct collapsed under a compression force. No fibrosis or vessel ingrowth were found in the PEG/PCL hydrogel after 4 weeks of ectopic cultivation, whereas the auricular chondrocytes showed proteoglycans' accumulation and collagen type II production. Rabbit auricular chondrocytes could survive and retain chondrogenic ability in the PEG/PCL hydrogel under both *in vitro* and *in vivo* conditions. While the PEG/PCL hydrogel did not show sufficient mechanical properties for supporting the cylindrical shape of the construct, the high chondrogenesis level of chondrocytes in the PEG/PCL hydrogel displayed the potential of this material for tracheal tissue engineering.

Keywords: Auricular chondrocytes, tissue engineering, trachea, cartilage, poly(ε-caprolactone), poly(ethylene glycol), hydrogel.

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Introduction

The concerns for patients with tracheal diseases are mainly about low quality of life and poor prognosis due to limited available options for reconstruction. While an artificial prosthesis or a composite graft can be chosen for tracheal reconstruction, clinical problems such as airway obstruction, bacterial infection or immune rejection may occur postoperatively (Dodge-Khatami *et al.*, 2003; Kim *et al.*, 2004; Kobayashi *et al.*, 2010; Teramachi *et al.*, 1997). Tracheal replacement is also difficult to achieve because of the shortage of donor tissue. Tracheal tissue engineering, which can bypass such limitations by using fully autologous cells, is an alternative option for tracheal reconstruction (Fishman *et al.*, 2014). Studies in tracheal tissue engineering focus on growth and chondrogenesis of chondrocytes that are cultivated with a variety of natural or synthetic materials (Law *et al.*, 2016). Ideally, a tissue-engineered cartilage should provide mechanical strength to maintain the cylindrical structure of

the airway. However, a perfect tissue-engineered cartilage construct has still to be developed and the neo-trachea is still suboptimal for clinical use. To tissue-engineer a tracheal cartilage, new materials need to be tested to understand the performance of the chondrocytes in different environments.

Poly(ε-caprolactone) (PCL) is used as a scaffold for cartilage tissue engineering because of its strong mechanical characteristics, slow degradation rate, non-toxic degradation products and good bio-compatibility (Lin *et al.*, 2008). Studies show that PCL is an ideal material for tracheal tissue engineering (Gao *et al.*, 2017; Tsao *et al.*, 2014). However, while PCL can be used to develop a three-dimensional (3D) scaffold, the seeded cells still grow on the surface of the material in a two-dimensional manner. A long period of *in vitro* or *in vivo* ectopic cultivation would be required for chondrocyte growth and cartilage formation. To overcome this limitation, a hydrogel scaffold can be considered since the cells can be mixed homogenously within the gel and grow in a 3D framework (Bryant and Anseth, 2002). Cell cultivation in hydrogels increases cell-material and cell-cell interactions, resulting in the decrease of chondrogenic de-differentiation (Benya and Shaffer, 1982; Bryant *et al.*, 2005; Moore *et al.*, 2006; Oudega *et al.*, 2001). Interestingly, the hydrophobic PCL forms a hydrogel when synthesised as a block co-polymer with hydrophilic poly(ethylene glycol) (PEG) and, therefore, can be crosslinked by exposure to either chemicals or ultraviolet (UV) (Drury and Mooney, 2003). The PEG/PCL co-polymer demonstrates excellent bio-compatibility and bio-degradability and is widely used for drug delivery (Khodaverdi *et al.*, 2016; Luo *et al.*, 2016; Payyappilly *et al.*, 2014). Indeed, the PEG/PCL co-polymer is used in cartilage tissue engineering. Park *et al.* (2007) successfully cultivate rabbit knee articular chondrocytes into neocartilage using a PEG/PCL-based scaffold under both *in vitro* and *in vivo* conditions. Further, Ko *et al.* (2013) encapsulate articular chondrocytes within a PEG/PCL hydrogel and reveal that the formation of hydrophilic and hydrophobic regions in the PEG/PCL hydrogel promotes chondrocyte aggregation, leading to increased production and accumulation of collagen type II. They show a successful repair of a rabbit knee cartilage defect by injecting articular chondrocytes and bone marrow mesenchymal stem cells (BMSCs) with a PEG/PCL hydrogel (Ko *et al.*, 2016). These studies reveal the potential of the PEG/PCL hydrogel for developing neo-cartilage; however, there is no evidence showing that the PEG/PCL co-polymer can be used for the reconstruction of tracheal cartilage.

Autologous chondrocytes can be isolated from various regions of the body, such as the articular, costal or auricular cartilages (Nayyer *et al.*, 2012). However, tracheal reconstruction with articular or costal cartilage is invasive and aggressive for the patient (Ahmad *et al.*, 2015). The harvesting of costal cartilage may produce postoperative complication, such as pneumothorax (Lusk *et al.*, 1993). The use of articular cartilage is also infeasible for autologous chondrocyte isolation because of the limited availability of donor tissue and the concerns of secondary osteoarthritis at the donor site (El Sayed *et al.*, 2010). Instead, auricular cartilage is an ideal source of autologous chondrocytes because it can be easily harvested without any aggressive surgical process (Lusk *et al.*, 1993). Autologous auricular cartilage graft is used for repairing small tracheal defects in animals and humans, thus revealing its excellent integrability with the laryngotracheal tissue (Ahmad *et al.*, 2015; Gungor *et al.*, 2003; Jacobs *et al.*, 1999; Lusk *et al.*, 1993; Temiz *et al.*, 2010). Weidenbecher *et al.* (2008; 2009) show the development of a cartilage sheet and the fabrication of a well-vascularised neo-trachea for tracheal reconstruction using autologous auricular chondrocytes (ACs). However, this scaffold-free cartilage sheet requires 6 to 10 weeks for cartilage regeneration to provide an adequate mechanical support. The mechanical properties of this neo-trachea are insufficient to replace a segmental defect and maintain long-term patency. This suggests that the ACs are suitable for cartilage tissue engineering, but incorporation of the scaffold is still required to maintain the structure, especially for engineering a cylindrical neo-trachea.

To our knowledge, no study shows a tissue-engineered trachea with encapsulated ACs within a PEG/PCL hydrogel. Gao *et al.* (2017) show successful cultivation of ACs on a 3D-printed PCL scaffold for long-segment tracheal reconstruction. Tsao *et al.* (2014) cultivate ACs in a porous PCL scaffold and successfully generate a neo-trachea. In addition, they show that co-cultivation of ACs and BMSCs enhances the chondrogenesis level of the neo-trachea. The aim of this study was to test whether ACs survived and retained the chondrogenic ability in a PEG/PCL hydrogel. In addition, whether the co-cultivation of ACs and BMSCs enhanced chondrogenesis in the PEG/PCL scaffold was tested. Furthermore, an *in vivo* experiment was conducted to test whether the PEG/PCL hydrogel supported the hollow cylindrical structure of the construct and to evaluate the feasibility of using this material in tracheal reconstruction.

Materials and Methods

Synthesis of the PEG/PCL co-polymer

The PEG/PCL co-polymer was synthesised by ring-opening polymerisation based on a previous study by Ko *et al.* (2013). Briefly, PEG (molecular weight = 3,350 g/mol, Sigma-Aldrich) and PCL (molecular weight = 968 g/mol, Sigma-Aldrich) were stirred at 100 °C. Stannous octoate was added to the reaction mixture as a catalyst. The reaction device was sealed under vacuum after purging with nitrogen. Then, the

reaction mixture was heated to 160 °C for 24 h while stirring. The resultant product was dissolved with dimethyl sulphoxide and dialysed at 4 °C to remove unreacted monomers. The product was freeze-dried to remove the remaining organic solvent and water. The synthesised polymers were verified using nuclear magnetic resonance spectroscopy.

Preparation of the PEG/PCL hydrogel

The PEG/PCL co-polymer was dissolved with phosphate-buffered saline (PBS, pH 7.4) at 10 %, 20 % or 30 % (w/v) and sterilised overnight by UV exposure. The photo-initiator 2-Hydroxy-2-methyl-propiophenone (0.1 %, Sigma-Aldrich) was added and mixed. Subsequently, the macromer/initiator solution was poured into a hollow cylinder mould (8 mm in diameter and 4 mm in height) and exposed to UV light (365 nm) for 3 min to form the hydrogel.

Mechanical properties analysis

The mechanical properties of the constructs were determined according to Tsao *et al.* (2014). Briefly, the elastic moduli of the PEG/PCL hydrogel discs were measured by axial compression using the Instron Mini44 (Instron, Canton, MA, USA) equipped with a 500 N load cell. To evaluate the elasticity of the PEG/PCL neo-trachea for luminal collapse resistance, the constructs were tested after 4-week ectopic cultivation by lateral compression using the ElectroForce (TA Instruments, New Castle, DE, USA) with a 25 N load cell. 5 mm-long rabbit tracheae were used as controls. All compression tests were performed using a constant strain rate of 5 mm/min. The elastic modulus was determined from the slopes of stress *versus* the strain in the computed plots.

Animals

All animal procedures followed the Chang Gung Memorial Hospital (Taiwan) animal research guidelines. New Zealand white rabbits (≈ 2 kg) were obtained from the Taiwan Livestock Research Institute. For anaesthesia, the rabbits were intramuscularly injected with 1 mL/kg each of Zoletil 50 (Virbac, Carros, France) and Rompun 20 (Bayer Korea Ltd., Seoul, South Korea). At the end of the experiment, the rabbits were anaesthetised using 1 mL/kg each of Zoletil 50 and Rompun 20 and euthanised using 2 meq/kg of KCl.

Macroscopic evaluations

Rabbit abdominal skin was shaved and sterilised with 10 % beta iodine and surgery was performed in supine position. A 50 mm-long incision was made along the abdomen region. The 10 %, 20 % and 30 % PEG/PCL hydrogel discs were implanted under the upper abdominal wall and sealed with 6-0 PROLENE® suture (Ethicon). The incision was closed with 4-0 nylon suture. The animals were checked daily to monitor for postoperative complications. At 2 and 4 weeks postoperatively, the incision site was re-opened and the macroscopic evaluations were performed. Swelling, inflammation and necrosis of the peripheral tissue around the implant were inspected grossly to assess the bio-compatibility and toxicity of the PEG/PCL hydrogel. Deformation of the scaffold was evaluated based on the extent of hydrogel collapse.

Cell isolation and cultivation

The isolation of ACs was based on a previous study by Lee *et al.* (2011). The ear cartilage was harvested from euthanised rabbits. The minced cartilage was washed with calcium- and magnesium-free PBS (pH 7.4) and digested for 3 h at 37 °C with 0.2 % type I collagenase (Sigma-Aldrich) in PBS. After filtrating through a 70 μm strainer (Falcon®), the cells were washed with PBS and collected by centrifugation (290 ×*g*, 15 min). The collected cells were cultivated in chondrocyte growth medium [Dulbecco's modified Eagle's medium (DMEM) containing 10 % foetal bovine serum and 1 % antibiotic-antimycotic (ThermoFisher Scientific)] at 37 °C and 5 % CO_2 and the culture medium was changed every third day.

The bone marrow was isolated from the iliac bone of the anaesthetised rabbits using syringes with an 18-gauge needle and heparin (100 U/mL) was immediately supplemented. The BMSCs were separated by Histopaque®-1077 (Sigma-Aldrich). After washing with PBS, the BMSCs were cultivated at 37 °C and 5 % CO_2 in minimum essential medium (MEM) containing 10 % foetal bovine serum (FBS), 4 ng/mL basic fibroblast growth factor (bFGF) (Peprotech) and 1 % antibiotic-antimycotic. The medium was replenished on the third day to remove non-adherent cells and, then, changed every third day. The ACs and BMSCs were cryopreserved and thawed at passage 1 and expanded to passage 3 for experiments. Two batches of cells were isolated from two individual rabbits and the cells from different batches were not pooled or used in the same experiment.

Encapsulation of cells in the PEG/PCL hydrogel and cell viability evaluation

ACs and BMSCs were encapsulated within the 20 % PEG/PCL hydrogel at three different ratios: (1) 100 % chondrocytes; (2) 70 % chondrocytes and 30 % BMSCs; (3) 30 % chondrocytes and 70 % BMSCs. 1×10^7 cells/mL were mixed with 200 μL of 20 % PEG/PCL macromer/initiator solution and the hydrogel discs (8 mm in diameter and 4 mm in height) were formed by UV photocrosslinking. The discs were cultured at 37 °C and 5 % CO_2 in chondrogenic defined medium [DMEM with 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 5.33 μg/mL linoleic acid, 1.25 mg/mL bovine serum albumin, 100 μg/mL sodium pyruvate, 100 nm dexamethasone, 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 5 ng/mL transforming growth factor-β1 (TGF-β1) and 1 % antibiotics]. The medium was replenished every third day.

Table 1. Primers used in the RT-qPCR.

Gene	Forward primer	Reverse primer
Gapdh	CCATTCATTGACCTCCACTACATG	CGTACTGGGCACCAGCATC
Mki67	AGAGATGCCAGTCACCAGAACC	TGGTATTAGAAATGCAGGTCTTTGC
Eln	AGCCAAAGCTTCCCAGTTTG	TCCAAGGACGCCTCCAAG
Sox9	CACTCCTCGTCCGGCATG	ACGTCGCTGCTCAGCTCG
Acan	AGAGGCCGAGTGGATCCAG	TGTGACGCGTCCCTCTGTC
Col2a1	CAACAGCAGGTTCACCTATACCG	ACCGGTACTCGATGACAGTCTTG
Col1a1	ATGGTGCCAAGGGAGATGC	TTAGCACCGACAGCTCCAGG

Cell viability evaluation of the encapsulated cells was performed with a LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFisher Scientific) following the manufacturer's instructions. The live cells (green florescence) and dead cells (red florescence) were viewed using a confocal microscopy (TCS SP8X, Leica). The viable cells were calculated as the number of live cells divided by the total number of cells.

RNA analysis

Total RNA was extracted with TRIzol® Reagent (ThermoFisher Scientific) and RNeasy® Mini Kit (QIAGEN). The cDNA was synthesised from 0.5 μg total RNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The quantitative reverse transcription PCR (RT-qPCR) was performed using the SYBR® Green PCR Master Mix (Applied Biosystem) and the QuantStudio 5 Real-Time PCR system (ThermoFisher Scientific) with following thermal profile: 2 min at 50 °C, 10 min at 95 °C and 40 cycles each of 15 s at 95 °C (denaturation) and 60 s at 60 °C (annealing and extension). The relative mRNA levels were calculated using the $^{\Delta\Delta}$Ct method and *Gapdh* was used as reference gene in each reaction. The primers used are listed in Table 1.

Histological evaluation

The 5 μm-thick paraffin sections were deparaffinised in xylene and rehydrated in graded alcohol followed by Verhoeff's elastic stain (Abcam), safranin O stain (ScyTek Laboratories, Logan, UT, USA), immunohistochemical staining of collagen type II and immunofluorescence staining of vimentin and CD31. For immunohistochemical staining, antigen retrieval was performed by heating the slides in citrate buffer solution at 95 °C for 20 min. The sections were blocked by Antibody Diluent, Background Reducing (Dako) for 1 h. After incubation at 4 °C overnight with anti-collagen II antibody (1 : 150; ab3092, Abcam) in antibody diluent and three times washing with PBS, the slides were incubated with Dako REAL™ EnVision™/HRP, Rabbit/Mouse for 30 min. Finally, staining was visualised after incubation for 3 min at 25 °C with Dako REAL™ DAB + chromogen (K5007, Dako). A positive reaction resulted in a brown staining. For immunofluorescence staining, the blocked sections were incubated with anti-vimentin antibody (1 : 300; ab8069, Abcam) for targeting the fibroblasts of the fibrous tissue or with anti-CD31 antibody (1 : 300; ab24590, Abcam) for targeting the endothelial cells of the vascular tissue. After incubation at 4 °C overnight, the slides were washed with PBS and incubated with donkey anti-mouse IgG antibody (1 : 500; ab150109, Abcam) for 30 min at room temperature. The sections were washed with PBS and mounted in flourished mounting medium with DAPI (ab104139, Abcam). The images were obtained using a fluorescence microscope (Axio Scope.A1, Carl Zeiss).

Determination of DNA, glycosaminoglycans and total collagen

The samples were lyophilised and digested in 1 mL of digestion buffer (100 U/mL papain, 0.1 M sodium acetate, 0.2 M sodium chloride, 0.05 M EDTA-Na_2, 0.1 M cysteine-HCl, pH 6) at 60 °C for 24 h and the supernatants were collected for quantification of DNA, glycosaminoglycans (GAGs) and total collagen content. The content of DNA was quantified using a NanoDrop spectrophotometer (ASP-3700, ACT gene, Piscataway, NJ, USA). GAG content was determined using an assay with a dimethyl-methylene blue dye (Sigma-Aldrich) in a UV-Vis spectrophotometer at 525 nm. Chondroitin-6 sulphate from shark cartilage (Sigma-Aldrich) was used as a standard. To determine the total collagen content, the hydrolysate was mixed for 18 h at 110 °C with an equal volume of 6 N HCl. The mixture was lyophilised and resuspended for 24 h at 4 °C in 500 μL of assay buffer (5 mg/mL citric acid monohydrate, 12 mg/mL sodium acetate trihydrate, 3.4 mg/mL sodium hydroxide and 0.12 % glacial acid in deionised H_2O, pH 6.0). The samples (80 μL) were mixed for 20 min at room temperature with 80 μL of chloramine-T reagent (14.1 mg/mL chloramine-T, 26 % n-propanol, 2.7 mg/mL citric acid monohydrate, 6.4 mg/mL sodium acetate trihydrate, 1.8 mg/mL sodium hydroxide and 0.06 % glacial acid in deionised H_2O) and, subsequently, incubated for 15 min at 60 °C with 80 μL Erlich's reagent (3.75 g of dimethlyaminobenzaldehyde in 15 mL of n-propanol and 6.5 mL of perchloric acid). Absorbance was measured at 540 nm. The content of total collagen was determined by measuring the hydroxyproline content using 0.1 as ratio of hydroxyproline to collagen.

Ectopic cultivation of the PEG/PCL neo-trachea

The photocrosslinked PEG/PCL hydrogel with 100 % chondrocytes, 70 % chondrocytes plus 30 %

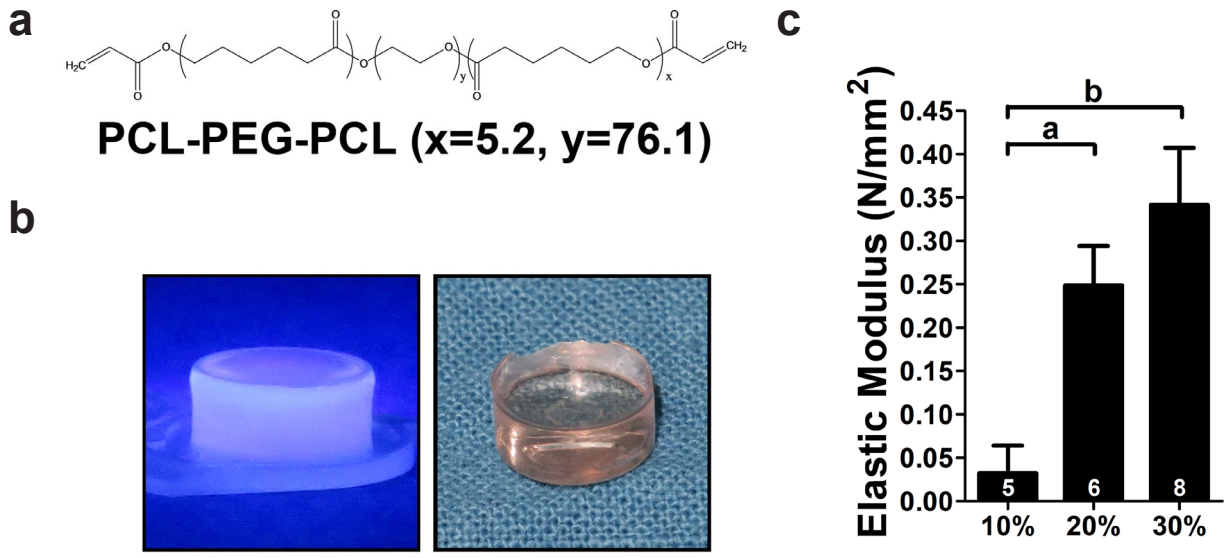

Fig. 1. Preparation of the PEG/PCL hydrogel. (**a**) Chemical structure of the PCL-PEG-PCL triblock co-polymer. (**b**) PEG/PCL hydrogel disc generated by UV photocrosslinking. (**c**) Elastic moduli obtained from stress-strain curves of unconfined compression tests on 10 %, 20 % or 30 % (w/v) PEG/PCL hydrogels. Data are expressed as mean ± SEM. n = 5, 6 or 8 hydrogels per group, as indicated on the data bars. [a] $p < 0.05$, [b] $p < 0.01$, Fisher's LSD test.

Table 2. Macroscopic evaluations. –, none; +, mild; ++, moderate; +++, severe.

			Peripheral tissue		
Gel concentration	**Week**	**Scaffold deformation**	**Swelling**	**Inflammation**	**Necrosis**
10 %	2	++	–	–	–
20 %	2	–	–	–	–
30 %	2	–	–	–	–
10 %	4	+++	–	–	–
20 %	4	–	–	–	–
30 %	4	–	–	–	–

BMSCs or 30 % chondrocytes plus 70 % BMSCs was fabricated and cultivated *in vitro* for 4 weeks before surgery. To mimic the shape of the trachea, the hydrogels were constructed with a 7 mm inner diameter, 12 mm outer diameter and 20 mm in length and two 2 mm C-shaped spaces were created. The abdominal skin of the anaesthetised rabbit was shaved and sterilised with 10 % beta iodine and the rabbit was positioned supine. A 50 mm-long incision was made along the abdomen region. By careful dissection and haemostasis, the *cutaneous maximus* muscle was elevated based on the superior epigastric vessels. Each construct was wrapped around with the vascularised *cutaneous maximus* muscle flap and a silicon rod (6 mm in diameter) was used to support the lumen during the *in vivo* cultivation. The incision was closed using 4-0 nylon sutures. In total, 5 mL of saline were administered intra-peritoneally to each rabbit. The animals were checked daily to monitor for postoperative complications. The PEG/PCL constructs were cultivated *in vivo* for 4 weeks.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance followed by Fisher's least significant difference (LSD) or Dunnett's multiple comparison test. Differences were considered statistically significant at $p < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Characterisation of the PEG/PCL hydrogel

To obtain the optimal hydrogel concentration for tracheal tissue engineering, the discs containing 10 %, 20 % or 30 % (w/v) PEG/PCL co-polymer were generated by UV photocrosslinking (Fig. 1**a**,**b**). With increasing concentration of PEG/PCL co-polymer, the elastic modulus of the hydrogel was increased (Fig. 1**c**). However, the elastic modulus of the 10 % PEG/PCL hydrogel was very low or undetectable, suggesting that the 10 % PEG/PCL hydrogel may

be insufficient to maintain the structure of the tissue-engineered construct. The 30 % PEG/PCL macromer solution largely lost its fluidity, limiting the homogeneity of the cells distributed in the gel. The 10 %, 20 % and 30 % PEG/PCL hydrogel discs were implanted under the *cutaneous maximus* muscle of the rabbits and the macroscopic evaluations were performed 2 and 4 weeks after implantation. The deformation of the 10 % PEG/PCL hydrogel was observed after 2 weeks of implantation (Table 2). In contrast, the disc structure of the 20 % and 30 % PEG/PCL hydrogels was maintained after 4 weeks of implantation, suggesting that the PEG/PCL hydrogel at concentrations above 20 % was sufficient to support the 3D space for cell growth *in vivo*. No swelling, inflammation or necrosis was observed in the peripheral tissues, revealing the bio-compatibility of the PEG/PCL co-polymer and its degradation products. Because the 20 % PEG/PCL hydrogel showed sufficient mechanical property and bio-compatibility, with an appropriate fluidity for mixing cells, it was chosen for the subsequent experiments.

Growth of ACs in the PEG/PCL hydrogels

Next, it was evaluated whether ACs could survive and proliferate in the PEG/PCL hydrogel. After 8 weeks of *in vitro* cultivation, the elastin was produced by ACs in the PEG/PCL hydrogel, as reflected by the Verhoeff's elastic stain (Fig. 2**a**). Approximately 100 % of ACs survived in the 20 % PEG/PCL hydrogel after 2 weeks of cultivation (Fig. 2**b**). This value was reduced to 66.9 % after 4 weeks of cultivation and to 62.5 % after 8 weeks. The DNA content did not increase during cultivation in the PEG/PCL hydrogel

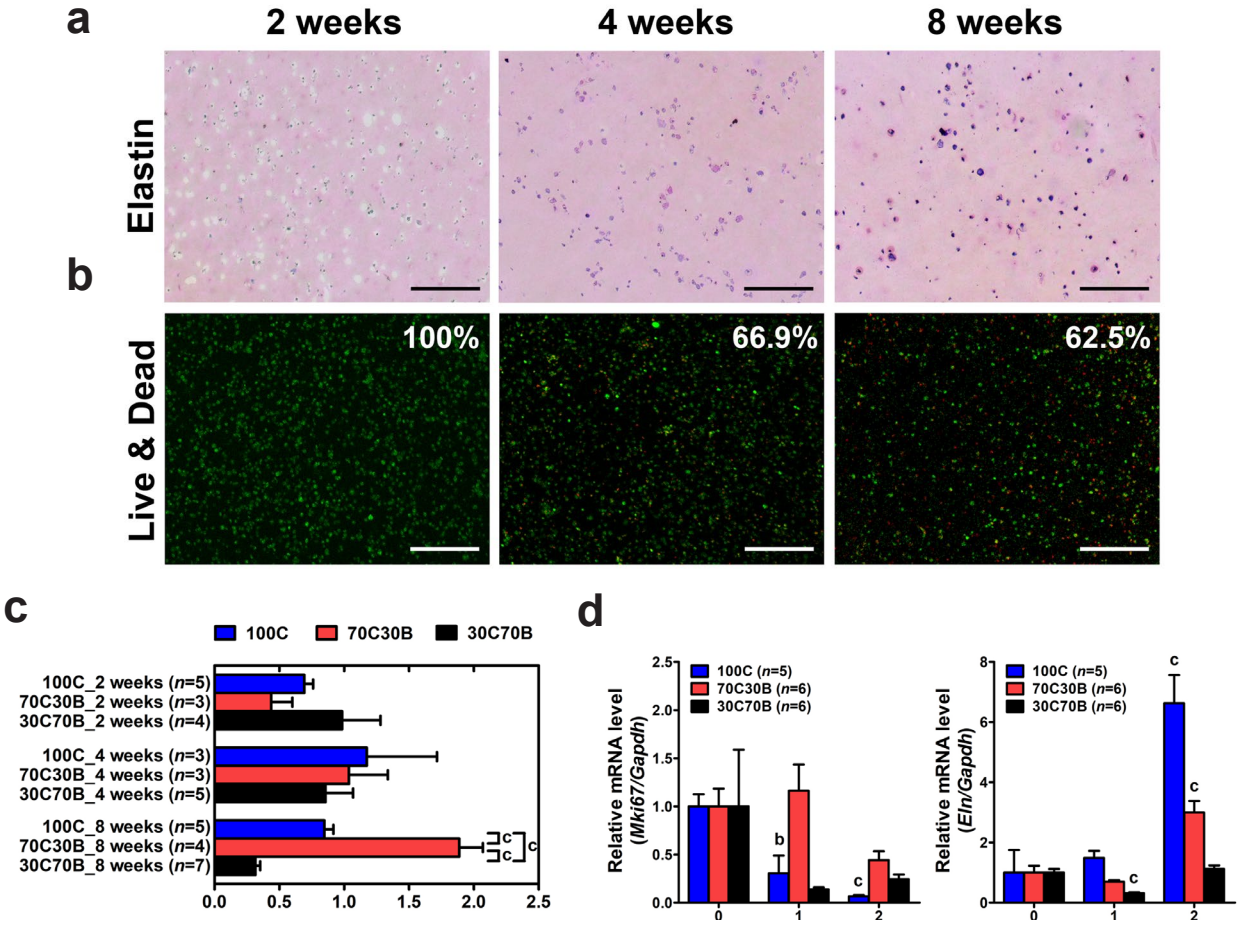

Fig. 2. Growth of ACs in the PEG/PCL hydrogel. (**a**) Verhoeff's elastic staining of ACs encapsulated in the 20 % (w/v) PEG/PCL hydrogel after 2, 4 or 8 weeks of *in vitro* cultivation. Scale bar = 250 μm. (**b**) Live and dead staining of ACs encapsulated in the 20 % (w/v) PEG/PCL hydrogel after 2, 4 or 8 weeks of *in vitro* cultivation. Green fluorescent staining indicates living cells, while red fluorescent staining indicates dead cells. The percentage of viable cells is shown in each panel. Scale bar = 250 μm. (**c**) DNA content in the 20 % (w/v) PEG/PCL constructs after 2, 4 or 8 weeks of *in vitro* cultivation. The data are expressed as mean ± SEM. n = 3, 4, 5 or 7 specimens per group, as indicated on the Y-axis. ^{c} $p < 0.001$, Fisher's LSD test. (**d**) Expression of *Mki67* and *Eln* in cells encapsulated in the 20 % (w/v) PEG/PCL hydrogel for 0, 1 or 2 weeks. mRNA levels are expressed relative to the average expression at day 0 in each group. Data are expressed as mean ± SEM. n = 5 or 6 per group, as indicated on the chart legend. ^{b} $p < 0.01$, ^{c} $p < 0.001$, Dunnett's test.

seeded with 100 % chondrocytes (100C) (Fig. 2**c**). The mRNA levels of the proliferation marker Ki67 (*Mki67*) decreased after 1 week of cultivation, as measured by RT-qPCR (Fig. 2**d**), whereas the ACs still expressed elastin (*Eln*) after 2 weeks of cultivation. These results suggested that the ACs were not proliferating but kept producing elastin in the PEG/PCL hydrogel.

To increase the living cells in the PEG/PCL construct, ACs were co-cultivated with BMSCs in various ratios. A time-dependent increase in DNA content was observed in the 70 % chondrocytes plus 30 % BMSCs group (70C30B), but not in the 30 % chondrocytes plus 70 % BMSCs group (30C70B) (Fig. 2**c**). While *Mki67* transcription decreased in the 70C30B group after 2 weeks (Fig. 2**d**), the highest *Mki67* mRNA level was observed in the 70C30B group as compared with the 100C and 30C70B groups during the 2-week cultivation period. In addition, the mRNA expression levels of *Eln* in the 70C30B and 30C70B groups were correlated with their ACs ratios, suggesting that the ACs maintained the expression of elastin in the presence of BMSCs.

Chondrogenic gene expression of the cell-encapsulated PEG/PCL hydrogel

The mRNA levels of the chondrogenic transcription factor Sox9 (*Sox9*), cartilage-specific proteoglycan core protein aggrecan (*Acan*) and cartilage extracellular peptide collagen type II (*Col2a1*) were significantly increased in the 100C group after 2 weeks of *in vitro* cultivation (Fig. 3). Already after 1 week of cultivation, *Sox9*, *Acan* and *Col2a1* were increased in the 70C30B group to values not observed in the 100C group at the same time point. Moreover, the expression level of these chondrogenic genes continuously increased in the 70C30B group after 2 weeks of cultivation. The 30C70B group also showed higher expression level of chondrogenic genes as compared the 100C

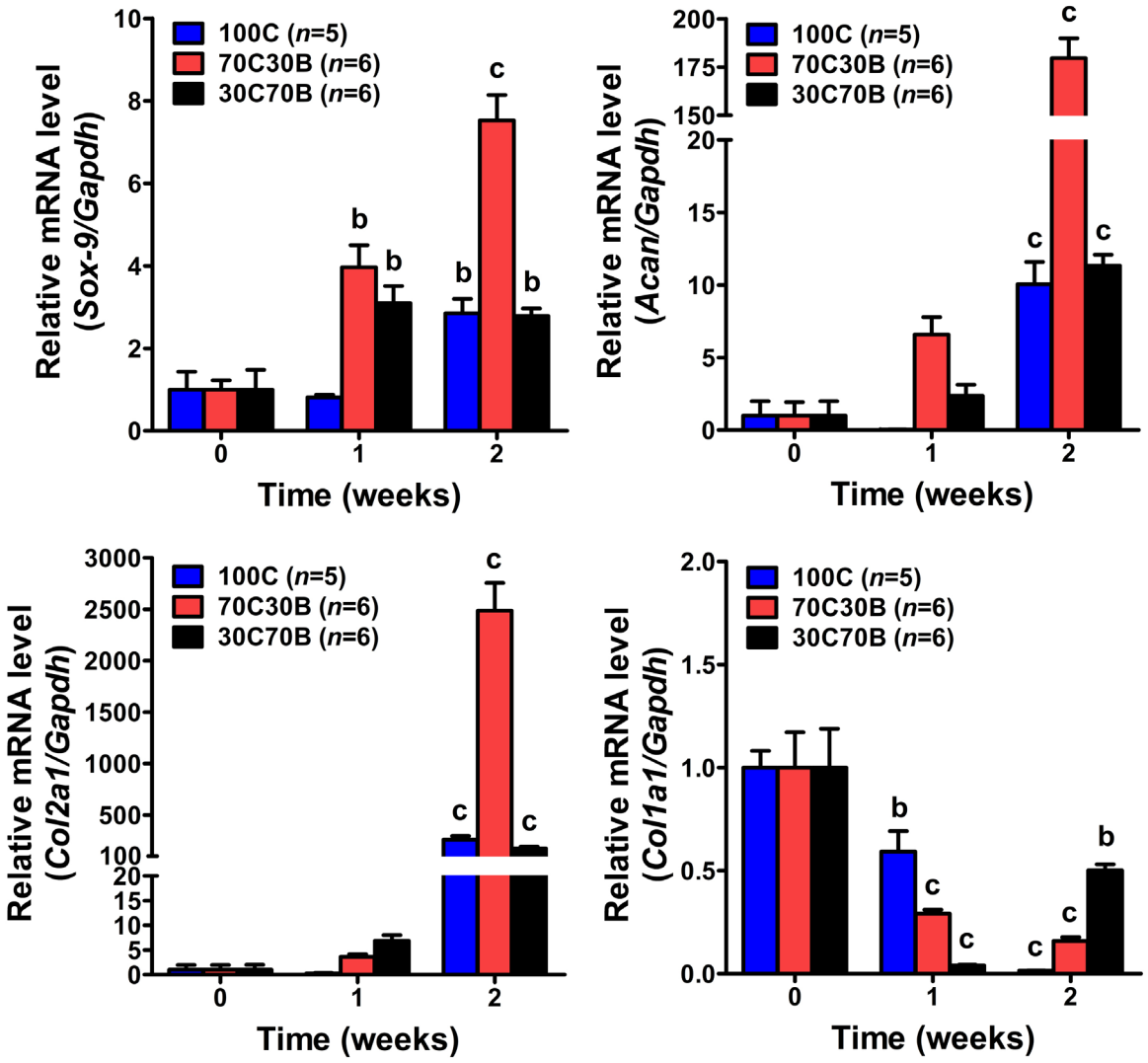

Fig. 3. Expression of *Sox9*, *Acan*, *Col2a1* and *Col1a1* in cells encapsulated in the 20 % (w/v) PEG/PCL hydrogel for 0, 1 or 2 weeks. mRNA levels are expressed relative to the average expression at day 0 in each group. Data are expressed as mean ± SEM. $n = 5$ or 6 specimens per group, as indicated on the chart legends. [b] $p < 0.01$, [c] $p < 0.001$, Dunnett's test.

group after 1 week. However, the expression of chondrogenic genes in the 30C70B group did not reach the levels of the 70C30B group after 2 weeks of cultivation. In addition, no chondrogenic de-differentiation was observed in the 100C, 70C30B and 30C70B groups during cultivation, as reflected by the decreased mRNA expression level of collagen type I (*Col1a1*).

Cartilaginous matrix expression of the cell-encapsulated PEG/PCL hydrogel

Safranin O staining showed proteoglycan accumulation in the 100C, 70C30B and 30C70B groups after 4 weeks of cultivation, with proteoglycan levels continuously increasing after 8 weeks of cultivation (Fig. 4**a**). In all groups, proteoglycans accumulated around the cells after 8 weeks of cultivation, with a large increase in the 70C30B group. Similarly, a time-dependent increase in GAG content was observed in all groups (Fig. 4**b**). The 70C30B group showed higher levels of GAGs on week 4 and week 8 than the 100C and 30C70B groups. In contrast, no increase in GAG content was found in the 30C70B group as compared with the 100C group during the 8-week cultivation. In addition, the GAGs to DNA ratio was determined to evaluate the GAG production per cell (Fig. 4**c**). While a similar GAGs to DNA ratio was found in all groups on week 8, the cells in 70C30B group showed the largest production of GAGs on week 4.

The accumulation of collagen type II in the PEG/PCL constructs was evaluated by immunohistochemical staining. After 4 weeks of cultivation, the collagen

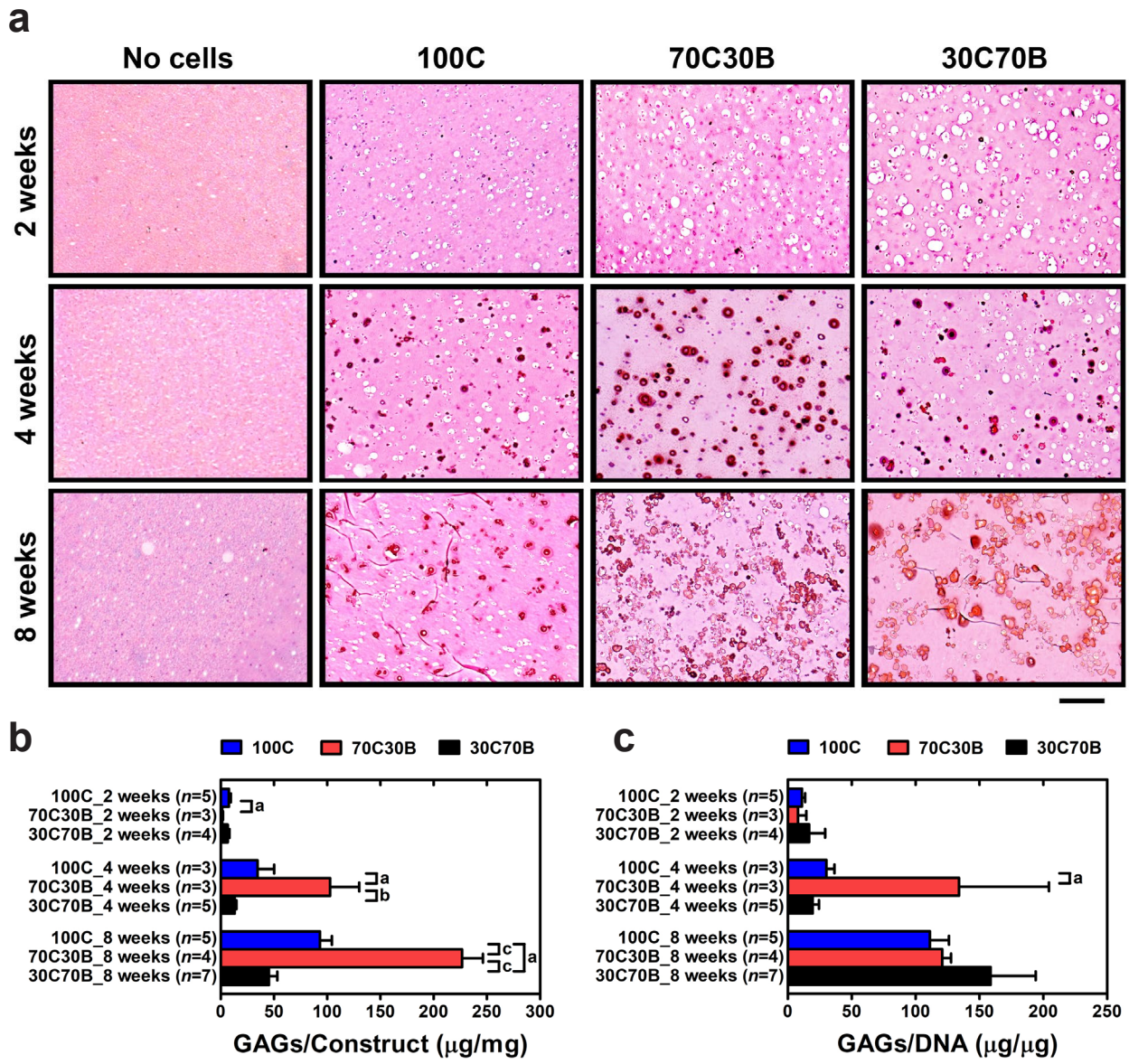

Fig. 4. Proteoglycan production in the PEG/PCL constructs. (**a**) Histological evaluation of proteoglycan accumulation in the 20 % (w/v) PEG/PCL constructs using safranin O staining after 2, 4 or 8 weeks of *in vitro* cultivation. Scale bar = 250 μm. (**b**) GAG content in the 20 % (w/v) PEG/PCL constructs after 2, 4 or 8 weeks of *in vitro* cultivation. Data are expressed as mean ± SEM. n = 3, 4, 5 or 7 specimens per group, as indicated on the Y-axis. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$, Fisher's LSD test.

type II was stained intracellularly in all groups (Fig. 5**a**). After 8 weeks of cultivation, the 100C group still showed an intracellular staining pattern of collagen type II in contrast to an extracellular collagen staining observed in 70C30B and 30C70B groups. Similarly, only a slight increase in total collagen content per construct was determined in the 100C group after 8 weeks of cultivation (Fig. 5**b**). Total collagen content per construct significantly increased in the 70C30B group in comparison with the 100C and 30C70B groups at week 8. Moreover, during the entire cultivation, the total collagen to DNA ratio in the 100C group was lower than in the 70C30B and 30C70B groups (Fig. 5**c**). Surprisingly, the 30C70B group showed the highest level of total collagen to DNA ratio at week 8. This result suggested that other type of collagens in addition to collagen type II might have been increased in the 30C70B group.

In vivo evaluation of chondrogenesis of the cell-encapsulated PEG/PCL hydrogel

The development of a vascular system is critical for supporting nutrition or providing stimulations for cell growth and differentiation in the implanted construct (Law *et al.*, 2016). Tsao *et al.* (2014) present an approach for the engineering of vascularised neo-trachea based on the ectopic cultivation of PCL scaffolds wrapped around by a pedicle muscle flap.

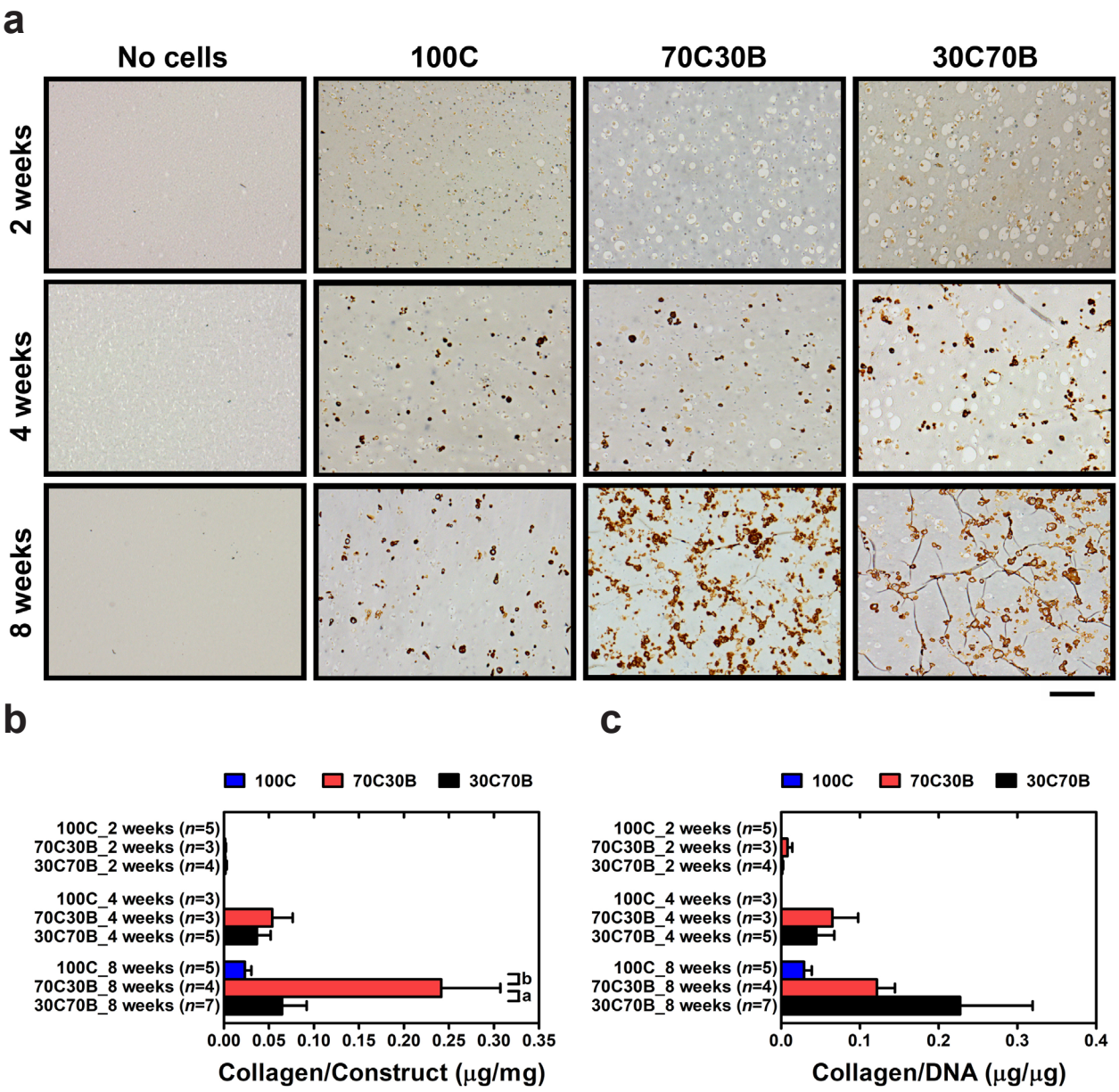

Fig. 5. Collagens production in the PEG/PCL constructs. (**a**) Immunohistochemical staining for collage type II in the PEG/PCL constructs after 2, 4 or 8 weeks of *in vitro* cultivation. Scale bar = 250 μm. (**b**) Total collagen content of the PEG/PCL constructs after 2, 4 or 8 weeks of *in vitro* cultivation. Data are expressed as mean ± SEM. $n = 3$, 4, 5 or 7 specimens per group, as indicated on the Y-axis. [a] $p < 0.05$, [b] $p < 0.01$, Fisher's LSD test.

Based on this approach, a 20-mm-long PEG/PCL construct (Fig. 6**a**) was generated and, subsequently, wrapped around with the rabbit *cutaneous maximus* muscle flap for the development of a vascularised neo-trachea (Fig. 6**b**). The silicon tube was used as a supporting stent and the entire neo-trachea was cultivated *in vivo* for 4 weeks (Fig. 6**c**). The neo-trachea maintained its cylindrical structure after 4 weeks of ectopic cultivation (Fig. 6**d**,**e**). However, the PEG/PCL hydrogel ruptured when subjected to a compression force (Fig. 6**f**). All neo-tracheae collapsed under a compression at 50 % strain and their elastic modulus at 20 % strain was largely lower than native trachea (Fig. 6**g**), revealing their insufficient mechanical strength. No fibrous or vascular tissue were found within the PEG/PCL neo-trachea, as reflected by the immunofluorescence staining of vimentin and CD31, respectively (Fig. 7). Moreover, the detachment of the PEG/PCL hydrogel and the peripheral muscle tissue occurred during the dehydration process of the histological procedure. These findings revealed that the PEG/PCL hydrogel did not entirely integrate with the peripheral muscle tissue. The proteoglycans were stained around the cells in the PEG/PCL hydrogel in the 100C group after 4 weeks of *in vivo* cultivation (Fig. 8). In contrast, fewer proteoglycans were stained

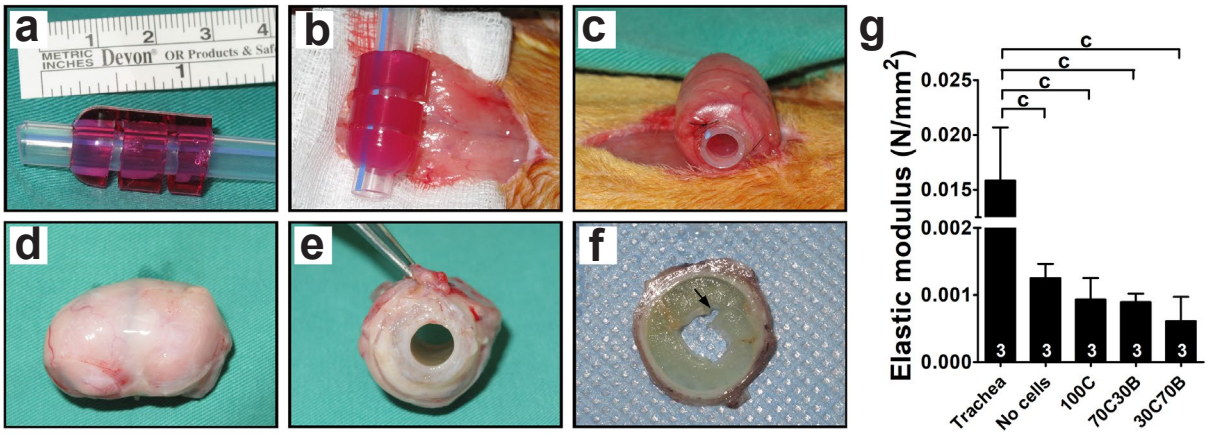

Fig. 6. Ectopic cultivation of the PEG/PCL constructs in rabbit. (**a**) The 20 mm-long PEG/PCL construct with two C-shaped gaps to mimic the shape of native trachea. (**b**) A vascularised *cutaneous maximus* muscle flap used to wrap around the constructs. (**c**) A silicone stent used to retain the cylindrical structure of the PEG/PCL neo-trachea. Gross appearance of the (**d**) external and (**e**) luminal surface of the PEG/PCL neo-trachea after 4 weeks of *in vivo* implantation. (**f**) Cross view of the PEG/PCL neo-trachea after compression with the forceps. The rupture site of PEG/PCL hydrogel is marked by an arrow. (**g**) Elastic moduli of rabbit trachea and PEG/PCL neo-trachea under lateral compression at 20 % strain. Data are expressed as mean ± SEM. $n = 3$ specimens per group, as indicated on the data bars. ^{c} $p < 0.001$, Fisher's LSD test.

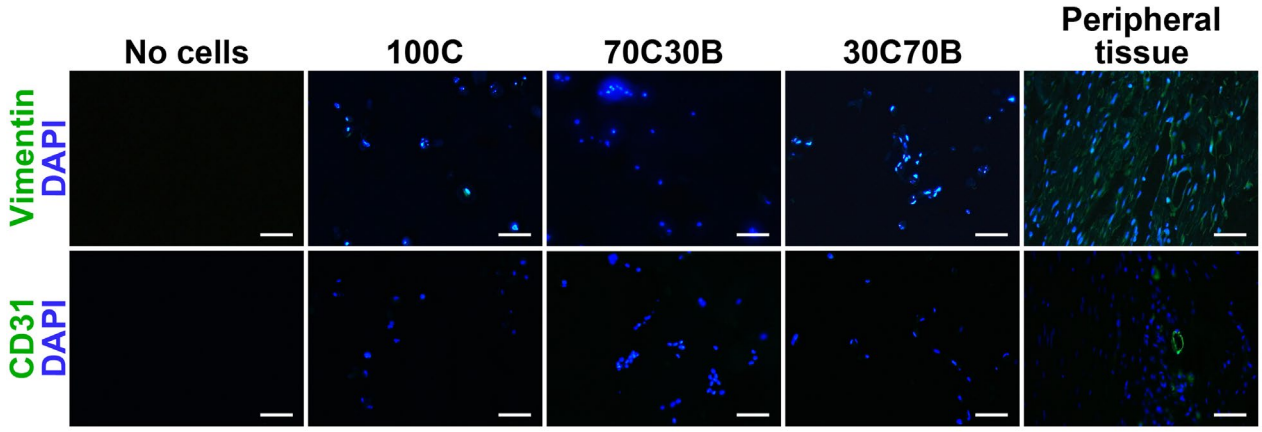

Fig. 7. Immunofluorescence staining of vimentin and CD31 in the PEG/PCL neo-trachea after 4 weeks of ectopic cultivation. The fibroblasts of the fibrous tissue and the endothelial cells of the vessel were immunolabelled with antibodies against vimentin (green, top row) and CD31 (green, bottom row), respectively. Cell nuclei were stained with DAPI (blue). The peripheral tissue was used as a positive control of staining. Scale bar = 50 μm.

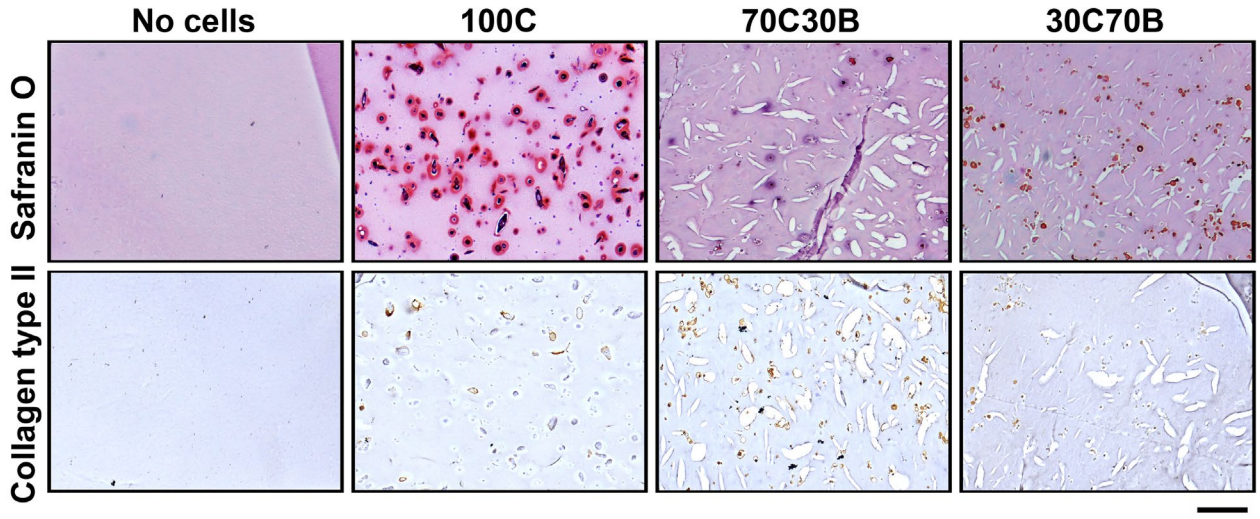

Fig. 8. Histological evaluation of the cartilaginous matrix in the PEG/PCL neo-trachea after 4 weeks of ectopic cultivation. The expression of proteoglycans (top row) and collagen type II (bottom row) are measured by safranin O staining and immunohistochemical staining, respectively. Scale bar = 250 μm.

in the PEG/PCL hydrogel in the 70C30B and 30C70B groups. Immunohistochemical staining of collagen type II showed only scattered positive cells in the PEG/PCL construct of the 100C group. While the cells expressed collagen type II in 70C30B and 30C70B groups, less extracellular collagen was found.

Discussion

Autologous chondrocytes can be isolated from various parts of the body. However, it is necessary to consider whether the process of chondrocytes isolation and the area of surgery are feasible, to avoid unnecessary damage or unwanted pain to the patients (Mossad and Youssef, 2009). Auricular cartilage is widely used as graft donor site for reconstruction procedures and the postoperative morbidity associated with auricular cartilage harvest is very low (Mischkowski *et al.*, 2008). Gao *et al.* (2017) prove that ACs can be cultivated on the surface of PCL to tissue-engineer a cartilage-like layer. However, this cartilage-like layer is eroded by peripheral tissue or immunocytes, suggesting that the cartilage-like layer or chondrocytes may require protection to avoid the damage from peripheral tissue. The present study demonstrated that the rabbit ACs remained alive and retained the chondrogenic ability in the 20 % PEG/PCL hydrogel under *in vitro* or *in vivo* conditions. Chondrogenic genes, including *Sox9*, *Acan* and *Col2a1*, were up-regulated in ACs after 2 weeks of *in vitro* cultivation in PEG/PCL hydrogel and the accumulation of proteoglycans in the construct was observed after 4 weeks of *in vitro* cultivation. The ACs continuously produced proteoglycans after the additional 4 weeks of *in vitro* or *in vivo* cultivation. This was similar to the results obtained by Ko *et al.* (2013; 2016), who demonstrate that the rabbit knee articular chondrocytes embedded in a 10 % PEG/PCL hydrogel express chondrogenic genes and produce GAGs after 2 weeks of *in vitro* cultivation. The two types of chondrocytes, auricular and articular, isolated from the elastic and hyaline cartilage, respectively, sustain their chondrogenic phenotype in the PEG/PCL hydrogel, suggesting that the PEG/PCL hydrogel is an ideal matrix for cartilage formation.

The hydrophilic PEG exhibits hydrogel properties, providing a highly swollen 3D structure with a high-water content and developing a tissue-like environment that is beneficial for promoting chondrocytes proliferation and maintaining chondrogenic functions (Bryant and Anseth, 2002; Ko *et al.*, 2013; Park *et al.*, 2007). On the other hand, the hydrophobic PCL provides mechanical strength and induces chondrocytes aggregation, thereby enhancing the production of cartilaginous matrix (Ko *et al.*, 2013). Thus, both the hydrophilic PEG and hydrophobic PCL contribute to the chondrogenesis in the PEG/PCL hydrogel. Importantly, PEG and PCL are Food and Drug Administration (FDA)-approved materials (Gong *et al.*, 2009). While the PEG/PCL co-polymer is not yet approved for clinical use, studies demonstrate that the PEG/PCL co-polymer is bio-compatible, with no toxicity to cells (Fu *et al.*, 2016; Gong *et al.*, 2009; Jung *et al.*, 2015; Ko *et al.*, 2016; Luo *et al.*, 2016; Park *et al.*, 2007). In the present study, approximately 100 % of ACs survived in the 20 % PEG/PCL hydrogel after 2 weeks of *in vitro* cultivation and no toxicity responses, such as swelling, inflammation and necrosis, were observed in the peripheral tissue around the PEG/PCL implants after 4 weeks of *in vivo* implantation. This evidence displays the safety of the PEG/PCL hydrogel for clinical use. PEG/PCL hydrogels are used in drug

delivery (Khodaverdi *et al.*, 2016; Luo *et al.*, 2016; Payyappilly *et al.*, 2014), bone regeneration (Fu *et al.*, 2012; Ni *et al.*, 2014) and knee cartilage repairment (Ko *et al.*, 2016). The present study illustrated the potential of PEG/PCL for developing a neo-cartilage construct using ACs for tracheal tissue engineering.

Chondrocytes stimulate the chondrogenic differentiation of BMSCs (Kang *et al.*, 2012; Ko *et al.*, 2016; Mo *et al.*, 2009; Tsao *et al.*, 2014). The present study showed that incorporation of 30 %, but not 70 % BMSCs, enhanced the expression of chondrogenic genes and increased the accumulation of GAGs and collagen type II under *in vitro* conditions. In comparison with 30 % chondrocytes, 70 % chondrocytes might produce higher levels of chondrogenic cytokines and stimulations, therefore providing a more adequate environment for the chondrogenesis of BMSCs. However, under *in vivo* conditions, the PEG/PCL construct composed of 70 % chondrocytes and 30 % BMSCs did not produce more cartilaginous matrix than the PEG/PCL construct composed of 100 % chondrocytes. This was likely due to the difference in nutritional requirement between chondrocytes and BMSCs. The nutritional requirements of chondrocytes are relatively low because the natural cartilage grows in a high cell density, low oxygen content and minimal blood supply environment (Strobel *et al.*, 2010; Zuscik *et al.*, 2008). In contrast, the BMSCs, which have a higher proliferation rate than chondrocytes, are sensitive to serum and oxygen deprivation (Potier *et al.*, 2007). While the nutrition is abundant in the culture medium, it is relatively low in the tissue fluid, which in turn, limits the growth and chondrogenesis of BMSCs during *in vivo* cultivation. In addition, the implanted PEG hydrogel does not integrate well with the native tissue (Liu *et al.*, 2011). In the present study, absence of vessels ingrown into the PEG/PCL hydrogel was observed, which may limit the nutrition supply in the central region of the construct. Thus, the chondrogenesis of BMSCs in the PEG/PCL hydrogel was likely dependent not only on stimulations from the ACs, but also on the adequate nutrition supply during cultivation. Park *et al.* (2007) use a porous PEG/PCL hydrogel generated by the salt-leach method for cartilage tissue-engineering, despite the cells being seeded, but not encapsulated, on the surface of the hydrogel. Rice and Anseth (2007) use a lipase supplement for accelerating the degradation rate and increasing the interconnections of the PEG/PCL hydrogel. Isogai *et al.* (2005) and Park *et al.* (2015) employ the 3D computer-aided design/manufacturing technique for generating a network structure on the construct for tissue engineering. Nevertheless, further studies are required for improving the interconnection of the PEG/PCL hydrogel and, consequently, increasing the chondrogenesis level in the PEG/PCL construct.

The histological results showed that proteoglycans were secreted by ACs and accumulated in the 20 % PEG/PCL hydrogel after 4 weeks of *in vitro* cultivation; however, no accumulation of extracellular collagen type II was found in the PEG/PCL hydrogel after *in vitro* cultivation. This was unlikely due to insufficient chondrogenic stimulations since *Sox9* (an upstream transcription factor of *Col2a1*) mRNA was up-regulated during *in vitro* cultivation. ACs were able to express *Col2a1* mRNA in the 20 % PEG/PCL hydrogel after 2 weeks of *in vitro* cultivation and collagen type II protein was stained intracellularly after 4 weeks of *in vitro* cultivation, revealing no dysregulation in collagen type II expression. GAGs distribution was more homogenous than collagen type II in the PEG/PCL hydrogel. This could be attributed to the fact that proteoglycans are smaller than collagen fibres (Ko *et al.*, 2013). However, the current study showed that the extracellular collagen was developed in the PEG/PCL hydrogel if the ACs were *in vitro* co-cultivated with BMSCs. This result indicated that the collagen type II protein was diffusible in the 20 % PEG/PCL hydrogel and the lack of extracellular collagen in the ACs-encapsulated PEG/PCL hydrogel was unlikely due to the limited diffusion of collagen. Also, this result suggested that the collagen production was possibly elevated to a higher level through co-cultivation of chondrocytes and BMSCs. Co-cultivation of 70 % ACs and 30 % BMSCs also accelerated the chondrogenic gene expression in the 20 % PEG/PCL hydrogel. Thus, future studies should continue to stimulate a higher level of collagen type II in ACs for the development of extracellular cartilage network in a PEG/PCL hydrogel.

For clinical applications, the tissue-engineered tracheal cartilage should provide an appropriate mechanical strength. The presented data showed that the cylindrical structure of the 20 % PEG/PCL hydrogel was sustained after 4 weeks of *in vivo* cultivation if a luminal silicon stent was used. However, after removal of the stent, the construct collapsed when under a compressive force. This suggested that the mechanical strength of the 20 % PEG/PCL neo-trachea might be insufficient to receive the dynamic motion of the trachea during respiration. Flowability and injectability are the advantages of the PEG/PCL hydrogel. These properties are useful not only for *in situ* repair of knee cartilage damage, but also for the 3D printing of pre-fabricated tracheal construct prior to reconstruction. In addition, the PEG hydrogel can be coated on the surface of the PCL (Wang *et al.*, 2016). Thus, the mechanical weakness of the PEG/PCL hydrogel could be compensated by hybrid assembling with other materials that have sufficient mechanical strength. The results of the present study demonstrated that the PEG/PCL hydrogel could be used for encapsulation and cartilage formation of ACs both *in vitro* and *in vivo*. These findings supported the idea of using the PEG/PCL hydrogel for tracheal reconstruction, whereas the weakness in its mechanical property should be tested and improved.

Conclusions

In clinical practice, tracheal reconstruction is limited by the shortage of donor tissue and the insufficient quality of substitutes. In this study, it was demonstrated that the ACs, which can easily be isolated from the ear, were able to survive and remain chondrogenic in 20 % (w/v) PEG/PCL hydrogel. The PEG/PCL hydrogel could entirely embed the cells and provide the 3D space as a native tissue. However, the PEG/PCL hydrogel did not show sufficient mechanical properties for supporting the cylindrical shape of the construct; in addition, this hydrogel could not be integrated within the peripheral tissue. Thus, it was concluded that the PEG/PCL hydrogel still could not be used alone for tissue-engineering a tracheal construct. However, the high chondrogenesis level of ACs and/or BMSCs in the PEG/PCL hydrogel displayed the potential of this material for tracheal tissue engineering. By modifying the PEG/PCL hydrogel with a 3D printing technique, pore-forming process or hybrid assembling approach, it could be possible to develop a neo-tracheal cartilage in the future.

Acknowledgements

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Discussion with Reviewer

Andrea Barbero: Several tracheal cartilage substitutes are engineered by different research groups and prove to be adequate as implants for the repair of trachea stenosis in animal models. What would be the possible advantage(s) of using the proposed approach as compared to the ones described in other studies for trachea replacement in patients?

Authors: Development of a perfect tracheal substitute for tracheal reconstruction remains a great challenge because of the variety of factors that should be taken into consideration; however, it is difficult to meet with all the conditions simultaneously. Some studies use materials with strong mechanical property, but these materials lack flexibility and limit the neck motion (Boazak and Auguste, 2018, additional reference; Park *et al.*, 2015). Some studies apply the 3D printing technology for generating the scaffold (Gao *et al.*, 2017; Isogai *et al.*, 2005; Park *et al.*, 2015), but it is not practical in most hospitals due to the lack of proper equipment for 3D printing. In some studies, the chondrocytes are cultivated in the hydrogel (Hong *et al.*, 2012; Vega *et al.*, 2017, additional references); however, similarly to this study, the mechanical problem still needs to be solved. The present work revealed the advantages of the PEG/PCL hydrogel, such as the maintenance of high chondrogenic ability of chondrocytes, and the disadvantages, such as the poor mechanical strength for supporting the cylindrical structure of the neo-trachea. An ideal tracheal substitute would need to make good use of different properties of available materials and minimise the inevitable shortcomings. To achieve this goal, thorough research of different materials and cells should be completed and the assembly of different materials to compensate their weakness will be the next step.

Andrea Barbero: The authors discussed that the limited extent of matrix produced by the PEG/PCL construct composed of ACs and MSCs was likely due to the difference in nutrition requirement between ACs and MSCs. However, after 4 weeks of chondrogenic co-culture, the MSCs within the graft became differentiated into chondrocytes. Therefore, the MSC-derived chondrocytes and the ACs would have the same "nutrition requirements". Can the authors provide additional explanations of the inferior *in vivo* maturation of the ACs/BMSC as compared to the ACs constructs?

Authors: While BMSCs can be differentiated into chondrocytes, a different gene expression pattern between ACs and BMSCs is reported (Hellingman *et al.*, 2012, additional reference). Therefore, it is unlikely that the BMSC-derived chondrocytes have the same properties as the ACs. Indeed, the concern relating to BMSCs in cartilage tissue engineering is mainly due to the possibility of de-differentiation or trans-differentiation under *in vivo* cultivation (Tan and Hung, 2017, additional reference). While this study showed that the de-differentiation gene *Col1a1* was not up-regulated *in vitro* (Fig. 3), whether the BMSC-derived chondrocytes de-differentiate or trans-differentiate to other type of cells during *in vivo* cultivation was uncertain. In addition, the chondrogenic cytokine TGF-β1 was supplemented in the chondrogenic-defined medium for *in vitro* cultivation and was no longer supplied after *in vivo* implantation. The lack of growth factor supplement could be another explanation for the inferior *in vivo* maturation of the ACs/BMSCs as compared to the ACs constructs. To clarify the effect of the ACs/BMSCs co-cultivation in the PEG/PCL hydrogel, further gene expression analyses of BMSC-derived chondrocytes after *in vivo* implantation and continuous supplementation of TGF-β1 during *in vivo* cultivation are required.

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Editor's note: The Scientific Editor responsible for this paper was Mauro Alini.