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Original Article

Feasibility of *in situ* chondrogenesis for the entire umbilical cord in preliminary preparation for tracheal graft

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Abstract: Background: There remains a scarcity of both autografts and allografts for tracheal transplantation after long-segmental resection. Subsequently, tissue engineering has become a promising alternative for tracheal transplantation, which requires successful *in vitro* chondrogenesis. Methods: To optimize the protocol for *in situ* chondrogenesis using the pig-derived whole Umbilical Cord (UC) as the starting material, it must be performed without using the UC-multipotent stromal cell (MSCs) isolation procedure. Nevertheless, chondrogenic induction is performed under a variety of conditions; with or without TGF-β1 at different concentrations, and also in combination with either a rotatory or hollow organ bioreactor. The engineered explant sections were analyzed using various histochemical and immunohistochemical stains to assess the expression of chondrocyte markers. Cell viability was determined through use of the APO-BrdU TUNEL assay kit. Results: The results showed that culture conditions induced heterogeneous chondrogenesis in various compartments of the UC. Moreover, explants cultured with 10 ng/ml TGF-β1 under hypoxic (1% O_2) in combination with a bioreactor, significantly enhanced the expression of aggrecan and type II collagen, but were lacking in the production of Glycosaminoglycans (GAGs), as evidenced by alcian blue staining. We speculated that whole segment UCs allowed for the differentiation into premature chondrocytes in our tissue-engineered environments. Conclusion: This study has provided exciting preliminary evidence showing that a stem cell-rich UC wrapped around an anatomical tracheal scaffold and implanted in vivo can induce nodes of new cartilage growth into a structurally functional tissue for the repairing of long-segmental tracheal stenosis.

Keywords: Chondrogenesis, umbilical cords, multipotent stromal cell, tracheal grafts

Introduction

Currently, an adequate treatment option is not available for long-segment tracheal stenosis. Fatal tracheal stenosis typically involves damage of the structurally supportive tracheal cartilage. In most cases, segmentary resection of the damaged trachea and primary anastomosis can be performed, but such methods cannot be used to repair long-segmental tracheal stenosis. In the event of extensive tracheal resection, auto/allograft and prosthetic materials have been used [1-4]. However, no satisfactory reconstructive option is available for such procedures [5]. Therefore, tissue-engineering strategies have been extensively studied as a potential treatment option. Basically, the trachea is considered a "complex tissue"; therefore tracheal transplantation should not be equated with other transplanted organs (i.e. the heart, lung, liver and kidney), which are donor-predominant, and used in combination with a rejection and anti-rejection regimen dilemma [6].

Tissue engineering has become one of the major areas of endeavor in medical research dating back to the 1990s [7]. A sustainable tissue-engineered trachea is an alternative treatment option for long-segment tracheal resec-

tion [8]. In addition to the conventional tissue engineering approach, a new substantially different concept "*in situ* tissue engineering", has been put into use where a tissue is structured or fabricated not *in vitro* but *in vivo*, exploiting the potential of the living body for wound healing, and subsequently applied for the purpose of tracheal reconstruction [9]. The trachea is composed of many tissues, amongst which tracheal cartilage integrity is the most crucial for ventilation function. Veritably, repair of long tracheal defects using cryopreserved cartilaginous allografts has been applied in piglets while showing promising results [10].

Currently, bone marrow (BM)-multipotent stromal cell (MSC) with chondrogenic potential has been used for tracheal reconstruction in the 3D bioprinting of artificial trachea [11]. MSCs in pellet culture have been induced by TGF-β1 or other cytokines and growth factors [12], biophysical stimulation [13], hypoxic conditions [14] and the provision of a suitable 3D environment [15] in order to undergo chondrogenic differentiation. For tracheal reconstruction, MSCs that have already undergone chondrogenesis should be recovered and re-suspended in single-cell suspension for 3D bioprinting. Usually, the human Umbilical Cord (UC) in a full term neonate is approximately 50 centimeters long and about 2 centimeters in diameter. The Wharton's Jelly (WJ) of the UC contains mucoid connective tissue and abundant MSCs, here referred to as UC-MSCs [16], which can undergo chondrogenesis under appropriate conditions. The human UC has nearly the same diameter of the trachea as pigs and contains abundant UC-MSCs with chondrogenic potential, therefore making it a potential cartilage source for tracheal "*in situ* tissue engineering".

In the current study, we tested the feasibility of pig UC as the cartilage source for tracheal "*in situ* tissue engineering" in piglets. Specifically, whole sections of pig UCs were wrapped around a 3D-printed tracheal scaffold and cultured in a rotating bioreactor. The engineered tissues were then analyzed for cell survival, cell growth and the expression of chondrocyte markers. The results show that cells in pig UCs in regard to these preparations were alive and had successfully underwent chondrogenesis. Moreover, the hypoxic condition (1% O_2) or culture in a rotating bioreactor further induced the expression of chondrogenic proteins. Additionally, pig UCs one week after culture in these preparations can be used as a promising source for tracheal transplantation. To optimize the protocol for *in situ* chondrogenesis, use the pig-derived whole UC as the starting material. This study provides exciting preliminary evidence showing that a stem cell-rich UC wrapped around an anatomical tracheal scaffold and implanted *in vivo* can induce nodes of new cartilage growth into a structurally functional tissue, for the purpose of repairing long-segmental tracheal stenosis.

Materials and methods

Tissue harvest and disinfection

[Affidavit of Approval of Animal Use Protocol (Taichung Veterans General Hospital) The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) Approval No: La-1071538].

The piglet UC was acquired through vaginal delivery occurring at a swine ranch. The newborn piglet was captured in a clean basin to avoid it from landing on the ground. The end of the UC proximal to the piglet was clamped with an umbilical cord clip, with the UC being held until completion of delivery, prior to the cutting of its distal end. Disinfection procedures were then instantly performed. First, the UC was collected in sterile phosphate buffer saline (PBS, 10× without calcium, magnesium, Hyclone, SH30258.02) and stored at a low temperature prior to being transferred to the laboratory. It was then immersed in diluted 10% polvidine solution for two minutes, rinsed in 75% ethanol for two minutes and finally rinsed in sterile 9% normal saline for three minutes. The UCs were washed several times with sterilized 1× PBS until no cord blood retention was seen, as that could have possibly contaminated the samples. The processed pig UCs were then stored in sterile normal saline after disinfection.

Wharton's Jelly (WJ) acquisition and MSC expansion

UCs (~20 cm long, and 20 g in weight) were collected in a sterile container filled with 1× PBS solution. MSCs were extracted within 24 hours, followed by the UCs harvest according to the

following steps: First, a longitudinal incision of the cord was made while its edges were fixated with aseptic pins before the umbilical arteries and veins were carefully removed; the WJ was then peeled off from the UC, with the WJ pieces then transferred to a sterile tube and thoroughly washed three times with 1× PBS. The treated jelly was briefly digested with type II collagenase, and then with trypsin/EDTA (Gibco, 15400054). The digestion was either with 0.075% type II collagenase for 0.5 hours and 0.125% trypsin/EDTA for 0.5 hours, or with 2 mg/ml type II collagenase for 16 hours and 0.25% trypsin/EDTA for 0.5 hours, before proceeding to 5% CO_2 in a 37°C incubator. The digested WJ exfoliation was washed with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, 11965) without FBS, containing 5% 100X antibiotics (Gibco, 15240062; penicillin 10,000 U/ml, streptomycin 10,000 μg/ml, and amphotericin B 25 μg/ml); and then centrifuged at 1,800 rpm for 10 minutes at room temperature. The filtered cells were resuspended in DMEM solution, supplemented with 10% FBS and seeded into 75T culture flasks to initiate cultivation under 5% CO_2 inside a 37°C incubator. The MSCs were subcultured at ~80% confluence in order to prevent contact inhibition of growth and spontaneous differentiation.

Chondrogenic differentiation of chondrocyte pellets formation and explants culture in various TGF-β1 concentrations under normoxia and hypoxia (N = 3 in each group)

Chondrocyte Induction Medium (CIM) was formulated with the following: αMEM (Gibco, 12561) containing 10^{-1} μM dexamethasone (Sigma, D1756), 50 μg/ml ascorbic acid-2 phosphate (Sigma, A4403), 40 μg/ml L-proline (Sigma, P5607), 1% 100× ITS^+ (Corning, 354352), 5% 100× antimycotic (Gibco, 15240), and 10 ng/ml of TGF-β1 (Peprotech, 100-21). Explants were cultured with 1 cm sized pig UC sections, which were used as positive controls for growth on the whole section of the UCs. They were then placed in CIM containing 0, 10, 20 or 40 ng/ml of TGF-β1 under hypoxia (1% O_2), or normoxia (21% O_2) at 5% CO_2 and 37°C for 21 days (**Figure 1** Up panel).

3D printed tracheal scaffolds and bioreactor

The 3D printed tracheal scaffolds were harvested from a donated mock-up trachea of a freshly killed listed pig made available for ordinary sale (~100 kg, 6.5 months old). The specimen was stored in normal saline at 4°C within 24 hours after removal from the animal. The native pig trachea was then scanned using a 3D scanner (Road Ahead Technologies Consultant Corporation), and its data saved as Stereolithography (.STL) files. The available scaffold trachea (size: 120×25×3 mm^3) was printed with a ProJet MJP 3600 printer, based on the scanned files using the SOLIDWORKS software. A 3D printing bioreactor, very similar to a commercial hollow organ bioreactor, (InBreath 3D Bioreactor for Hollow Organs, Bronchus, Trachea & Blood Vessels) from Harvard Apparatus was used, with crystal (VisiJet® Crystal Plastic Material) as the material used for printing on a ProJet MJP 3600 without any bio-toxic effects. The Breath 3D-bioreactor has the rotating double-chamber of a tubular matrix and uses the rotator movement of the scaffold around its longitudinal axis. By immersing half of the construct in media at any one moment, tissues are alternately exposed to the gaseous and liquid (medium) phases. During exposure to the incubator's atmosphere, the construct remains wet, with the thin surface layers of the culture medium periodically oxygen-saturated [17].

Whole section of the UC wrapped around the tracheal scaffold (N = 3 in each group)

The fabricated tracheal scaffolds and assembled bioreactor were disinfected in 75% ethanol for 4 hours, and then overnight in 10% hydrogen peroxide. For evaluation of the bioreactor-driven physical force that had contributed to chondrogenesis, we used a 10 cm long whole section pig UC obtained through a longitudinal incision and had it wrapped around the tracheal scaffold. It was fixated by an interrupted suture over the edge for approximating the UC cut end, and then reinforced with a freehand tie over both ends, for the purpose of securing sliding between the UC and the scaffold. The preparation was then cultured in the hollow organ bioreactor at slow rotations (1 rpm/min), with a half chamber volume of CIM containing 10 ng/ml TGF-β1 at 5% CO_2 at a temperature of 37°C under normoxia for 21 days (**Figure 5** Up panel). During this period, we changed the culture medium every 2-3 days. The cultured specimens of the UC were stained for IHC, alcian blue, safranin O and TUNEL assay to evaluate chondrogenesis and cell viability.

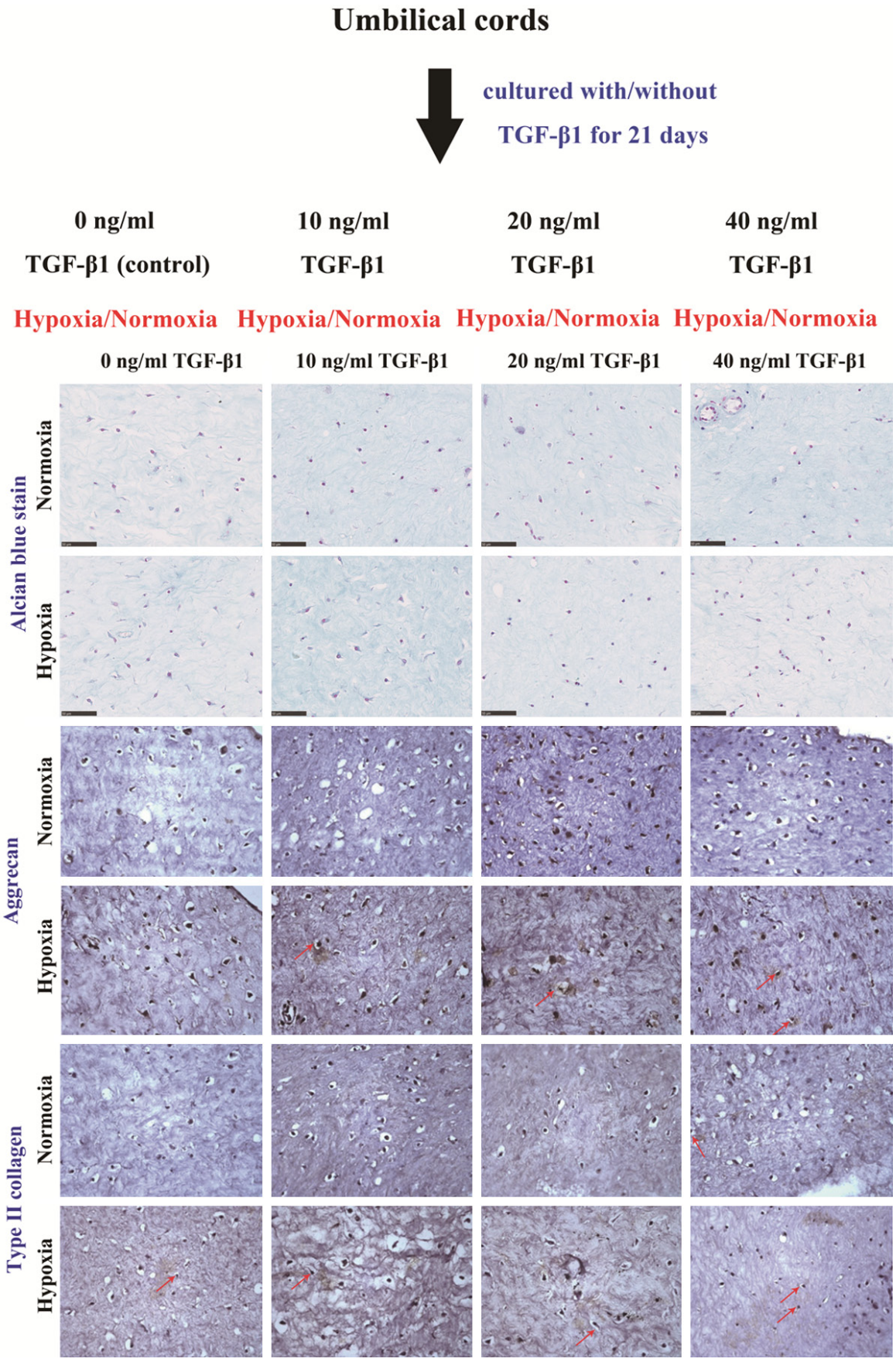

Umbilical cords
cultured with/without
TGF-β1 for 21 days
0 ng/ml
TGF-β1 (control)
10 ng/ml
TGF-β1
20 ng/ml
TGF-β1
40 ng/ml
TGF-β1
Hypoxia/Normoxia
Hypoxia/Normoxia
Hypoxia/Normoxia
Hypoxia/Normoxia
0 ng/ml TGF-β1
10 ng/ml TGF-β1
20 ng/ml TGF-β1
40 ng/ml TGF-β1
Alcian blue stain
Normoxia
Hypoxia
Aggrecan
Normoxia
Hypoxia
Type II collagen
Normoxia
Hypoxia

Figure 1. Porcine UC explants cultured in medium supplied 0, 10, 20 or 40 ng/ml of TGF-β1 under normoxia or hypoxia for 21 days, and the expression of aggrecan/type II collagen was analyzed by IHC, and GAGs with alcian blue stain. 1 cm umbilical cord tissues were cultured in a medium with TGF-β1 (0, 10, 20 or 40 ng/ml TGF-β1) under normoxia/hypoxia for 21 days. The expression of alcian blue/aggrecan/type II collagen was analyzed by IHC. Blue color indicates GAGs expression. Brown color indicates Aggrecan/type II collagen expression; red arrows indicate lacuna-like cells. Note color reaction products around the cell. Magnification ×400X.

Histochemical staining and Immunohistochemistry (IHC) for chondrogenic markers

The treated specimens were fixed in 10% formalin, and embedded in paraffin, with the paraffin sections (4 mm in thickness) subsequently used for evaluation. We then applied alcian blue staining for the Glycosaminoglycan (GAG) components. For the alcian blue stain, the specimens on the slides were deparaffinized and rehydrated, and then incubated in 3% acetic acid (Sigma, 33209) for 3 minutes. Following immersion in a pH2.0 alcian blue solution (Scy Tek, ANC250) for 30 minutes, the remaining dye was removed under running tap water for 5 minutes. Cell nuclei were stained with a 0.1% Nuclear Fast Red solution (Sigma, N3020) for 3 minutes prior to undergoing a thorough rinse. IHC with antibodies against aggrecan (Biorbyt, orb10066) and type II collagen (Abcam, ab-34712) were performed, using HRP Polymer and DAB Plus Chromogen (UltraVision LP Detection System, Thermo, TL-060-HD). After deparaffinization and rehydration, the slides were immersed in 0.1% PBST for 15 minutes. They were then incubated in 10 mM Sodium Citrate (pH6.0) for antigen retrieval at 96°C or a higher temperature for 10-20 minutes. After reacting with a Hydrogen Peroxidase Blocking solution for 10 minutes, an Ultra V Block solution was added for an additional 5 minutes. The final addition, using either aggrecan or a type II collagen antibody (1st antibody), allowed overnight reaction to occur at 4°C. The conjugated tissues were then rinsed a total of 3 times using 0.1% PBST, combined with the Primary Antibody Enchaner solution for 10 minutes. Finally, the preparation was reacted with HRP Polymer for 15 minutes in the dark, and DAB Substrate was acquired for quantification. For H&E stain, the protocol was the same as IHC prior to the 1st antibody reaction; the samples were wrapped up with eosin stain for 5 minutes subsequent to hematoxylin fixation for 2-3 minutes for the purpose of histological analysis.

APO-BrdU TUNEL assay in situ DNA fragmentation assay for apoptosis analysis (N = 3 in each group)

To evaluate the UC cells with DNA fragmentation, UCs were kept in the culture plates and treated with TGF-β1 at different levels (0, 10, 20 or 40 ng/ml), or a combination involving 2 types of bioreactors (a rotatory and hollow organ bioreactor). For the analysis of cell apoptosis, tissues were fixed with 10% formalin and the assays protocol was performed according to the manufacturer's instructions (Apo-BrdU in situ DNA fragmentation assay kit; BioVision, Inc., Milpitas, CA, USA). First, samples were deparaffinized and rehydrated, and then fixed in 4% formaldehyde in PBS for 15 minutes. Second, samples were incubated in a DNA Labeling Solution at 37°C in the dark for 60 minutes. Third, Antibody Solution was added to react with the samples in the dark for 30 minutes. Finally, Propidium Iodide/RNase, a staining solution was applied in the dark for 30 minutes. Apoptosis was quantitatively analyzed through use of laser confocal microscopy (Olympus FV1000).

Statistical analysis

Statistical analysis was performed in chondrogenesis in the WJ area of UC (lacuna-like cells as chondrocytes), for signal intensity in IHC, for alcian blue of GAG in extracellular matrix of UC, and for the number of decayed cellular nuclei detected with APO-BrdU TUNEL assay through artificial visual account. The number of chondrocytes was calculated using IHC images with obvious lacuna-like cells in a random selection of heterogeneous chondrogenesis of UC (N = 5/group), and indicated with + numbers from a low to high level (1~10 chondrocytes as +; 11~20 chondrocytes as ++; 21~30 chondrocytes as +++; 31~40 chondrocytes as ++++; >41 chondrocytes as +++++). IHC assay was selected as the prominent brown area of the signal (N≥4/group), and alcian blue stain was imaged with obvious lacuna-like cells (N≥4/

group). The signal intensity was indicated with - or + numbers from a low to high level (IHC/Alcian blue signal intensity: no detection as -; weak as +; moderate as ++; strong as +++). APO-BrdU TUNEL assay detected the merge area of both red and green fluorescent signals (Apo-BrdU inrtensity: 0~5 apoptotic cells as -; 6~30 apoptotic cells as +; 31~55 apoptotic cells as ++; 56-90 apoptotic cells as +++, N = 4/group). The above data of signal intensity were indicated with - or + numbers from a low to high level for purposes of comparison with pig native tracheal as the baseline group by three distinct professionals.

Results

We optimized the protocol for *in situ* chondrogenesis using the whole UC as a starting material without the UC-MSCs isolation procedure. Nevertheless, chondrogenic induction was performed under a variety of conditions: with or without TGF-β1 at different concentrations, and in combination with either a rotatory or hollow organ bioreactor. These conditions and assay outcomes are summarized in **Tables 1**, **2** (Number of chondrocytes and signal intensity indicated with - or + numbers from a low to high level for comparison with statistical significances under visual calculation due to the heterogeneous background). In all groups, lacuna-like cell growth was consistently observed. Additionally, the same results were noted under hypoxia with 0, 10, 20 or 40 ng/ml TGF-β1. Although this study has yielded a mixed pattern of results, it has shown the striking effect which 10 ng/ml TGF-β1 has on the acquisition of chondrocyte phenotypes. Thus, any subsequent UC cultures placed in a bioreactor were performed in the presence of 10 ng/ml TGF-β1 for the purpose of fundamental control.

Effects of TGF-β1, hypoxia and rotatory bioreactor culture on chondrogenesis

We detected the expression of aggrecan and type II collagen through the use of immunostaining in the UC explants cultured after 21 days, using 10, 20 and 40 ng/ml TGF-β1 under hypoxia, while only detecting weak alcian blue staining in the 10 ng/ml TGF-β1 culture groups (**Figure 1** Down panel). In general, the growth of lacuna-like cells was observed in all groups. Hypoxic cultures (1% O_2) particularly exhibited greater expressions of aggrecan and type II collagen than those of normoxic cultures. On the contrary, the same expressions of aggrecan and type II collagen were found regardless of the differences in TGF-β1 concentrations (10, 20 or 40 ng/ml TGF-β1) (**Figure 1** Down panel). Furthermore, the UC explants cultured under hypoxia over 21 days with 10 ng/ml TGF-β1 in combination with a rotatory bioreactor (**Figure 3** Up panel) increased in their expression of aggrecan and type II collagen within the subamnion, WJ and perivascular areas, while showing few changes in the quality and number of lacuna-like cells. This result is in tandem with alcian blue staining when compared with uncultured UC explants (**Figure 3** Down panel). Meanwhile, the UC explants exhibited an increase in apoptotic cells even within the rotatory bioreactor under hypoxia when compared to uncultured UC explants (**Figure 4**). These data when analyzed together suggest that TGF-β1 at 10 ng/ml, hypoxia and rotatory bioreactor culture all enhance the expression of chondrogenic proteins, such as aggrecan and type II collagen, in the UC explant culture.

Heterogeneous premature chondrocyte growth in cultured UC sections

Heterogeneous premature chondrocytes growth was detected by alcian blue, Safranin O and H&E stains within the subamnion, WJ and perivascular areas (Supplementary Figures 1, 2, 3). A 3D-printed tracheal modular scaffold was then covered with the whole section of a pig UC, which was then cultured in a hollow organ bioreactor for 21 days under normoxia (**Figure 5** Up panel). The alterations in the mechanical indices after 21 days included shrinkage of WJ with mass volume reduction, despite an intact ammonic membrane having the same elasticity as Day 0 (Supplementary Figure 4). IHC stains indicated a vivid lacuna-like cell formation and a wider range of aggrecan and type II collagen expressions, when compared with the uncultured UC (**Figure 5** Down panel). However, alcian blue stain still revealed a weak positivity for this chondrocyte marker in comparison with the uncultured UC. The quantity and morphology of the lacuna-like cells appeared superior to the UC culture alone without an adjunctive bioreactor (**Figure 1**). Based upon the positive staining of aggrecan in IHC, but the negative staining of alcian blue, we speculate that these primitive chondrocyte-like cells may not have

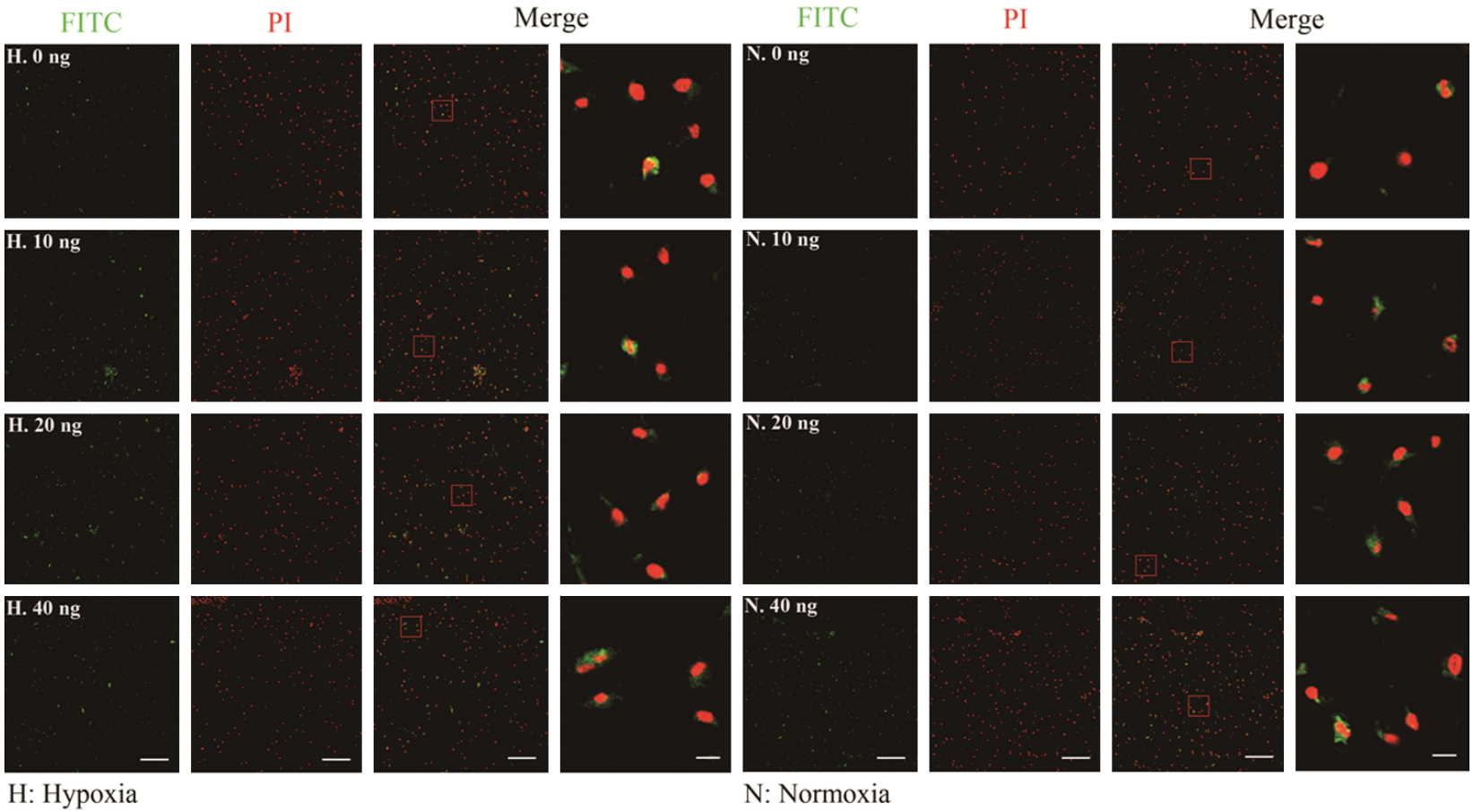


Figure 2. Apoptotic cells in porcine UC explants cultured within a medium supplied 0, 10, 20 or 40 ng/ml of TGF-β1 for 21 days under normoxia or hypoxia. FITC: Green fluorescence labeled cells indicate apoptotic signal (Apo-BrdU); PI: Red fluorescence labeled total cells; Merge: Merging green and red fluorescence. Lines 1-3 and 5-7 (scale bar = 100 μm with magnification ×200X); The merged data of FITC/PI was the same area as the red box in lines 3 and 7, and at high magnification in lines 4 and 8 (scale bar = 10 μm).

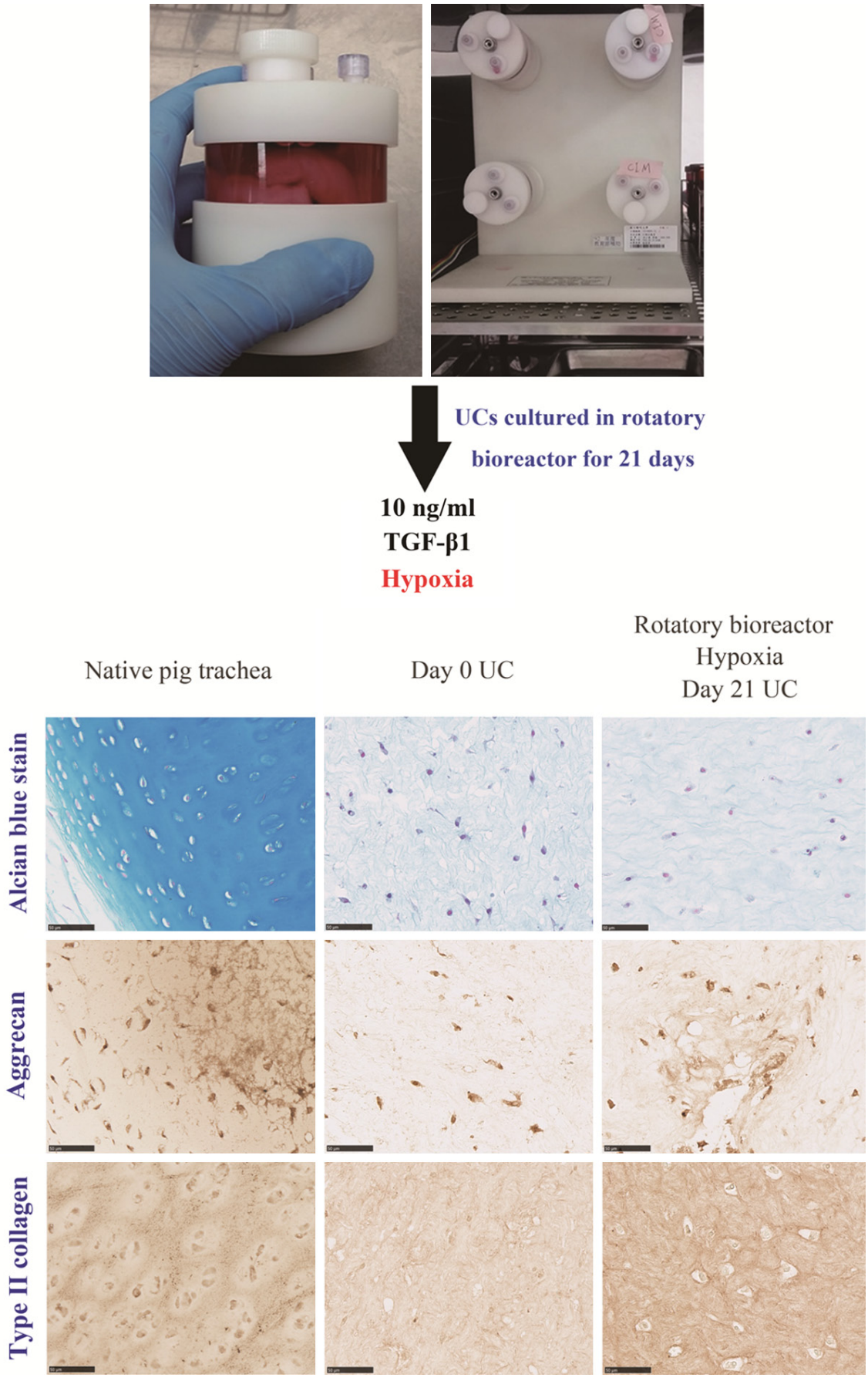

UCs cultured in rotatory bioreactor for 21 days
10 ng/ml
TGF-β1
Hypoxia
Native pig trachea
Day 0 UC
Rotatory bioreactor
Hypoxia
Day 21 UC
Alcian blue stain
Aggrecan
Type II collagen

Figure 3. The Umbilical Cord (UC) explants cultured within a rotatory bioreactor under hypoxia for 21 days, and the expression of aggrecan/type II collagen was analyzed by IHC, and GAGs with alcian blue stain compared with a native pig trachea and uncultured UC. 1 cm long umbilical cord tissues were cultured in a Chondrocyte Induced Medium (CIM) containing 10 ng/ml TGF-β1 within the rotatory bioreactor under hypoxia for 21 days. The expression of aggrecan/type II collagen increased after being cultured with a CIM containing 10 ng/ml TGF-β1 under hypoxia for 21 days, but there seems to be no increase in alcian blue stain. Blue color indicates GAGs expression. Brown color indicates Aggrecan/type II collagen expression. Magnification ×400X.

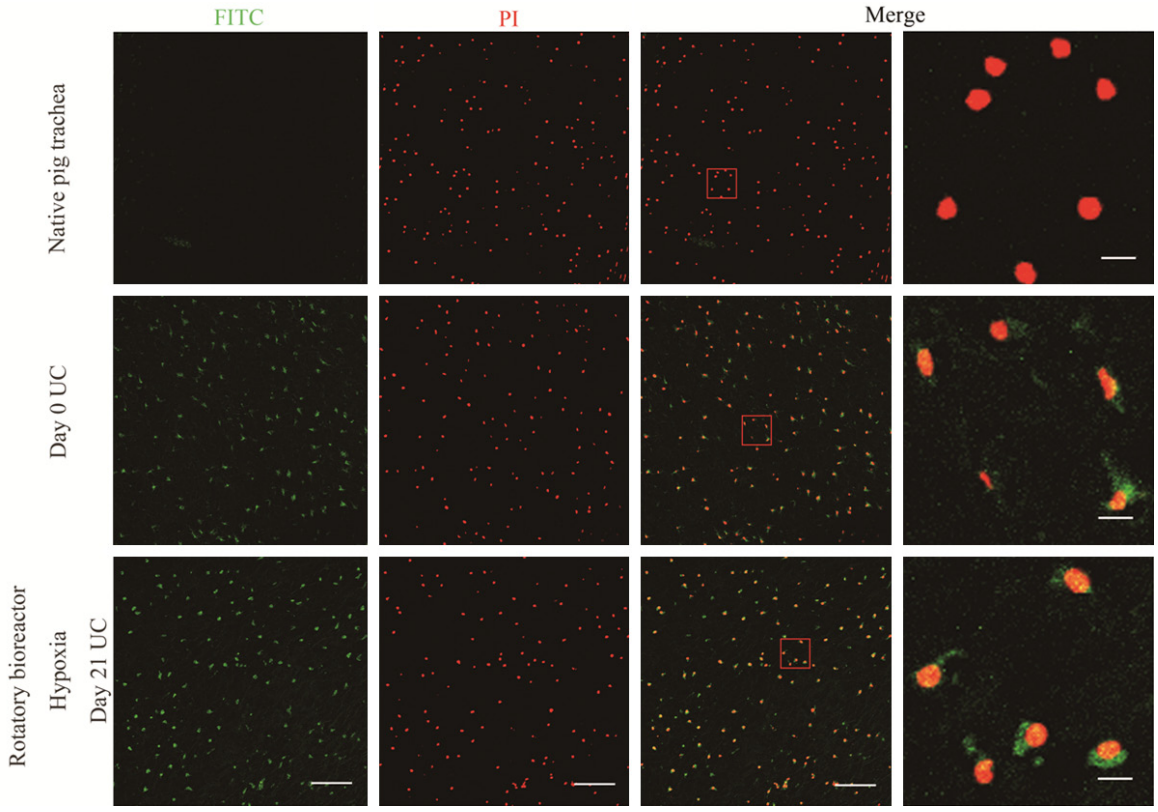
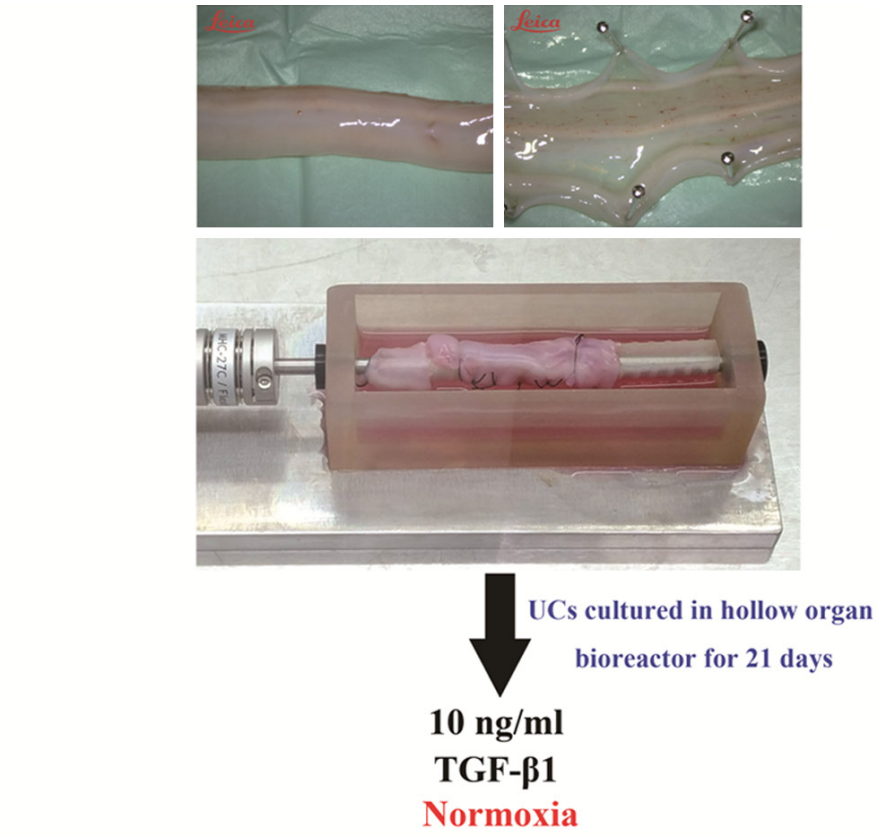


Figure 4. Apoptotic cells in porcine UC explants cultured within a rotatory bioreactor under hypoxia for 21 days compared with a native pig trachea and uncultured UC. 1 cm long umbilical cord tissues were cultured in a Chondrocyte Induced Medium (CIM) containing 10 ng/ml TGF-β1 within a rotatory bioreactor under hypoxia for 21 days. FITC: Green fluorescence labeled cells indicate apoptotic signal (Apo-BrdU); PI: Red fluorescence labeled total cells; Merge: Merging green and red fluorescence. Lines 1 to 3 (scale bar = 100 μm with magnification ×200X); The merged data of FITC/PI was the same area as the red box in line 3, and at high magnification in line 4 (scale bar = 10 μm).

undergone chondrogenesis at a level mature enough to secrete GAGs in the pericellular regions. This speculation was supported by the comparison made with the native pig trachea control, which revealed robust expressions of aggrecan and type II collagen in both IHC and alcian blue staining (**Figure 5** Down panel).

Escalating cell apoptosis occurred in UC explants cultured under miscellaneous conditions

To determine and compare the degrees of cell apoptosis in the pig UC explants cultured under different conditions, paraffin sections from different culture conditions were analyzed using the "Apo-BrdU *In Situ* DNA Fragmentation Assay Kit". We also compared the numbers of apoptotic cells of each culture condition with fresh native pig tracheal cartilage (negative control) and D0 UC explants. We detected strong apoptosis in the D21 UC explants cultured in the presence of TGF-β1 at different concentrations (10, 20 or 40 ng/ml TGF-β1) (**Figure 2**). Meanwhile, the combination of a rotatory bioreactor under hypoxia (**Figure 4**), or hollow organ bioreactor under normoxia (**Figure 6**) did not reduce the apoptosis event when compared with the fresh native pig trachea sections (con-


UCs cultured in hollow organ
bioreactor for 21 days
10 ng/ml
TGF-β1
Normoxia

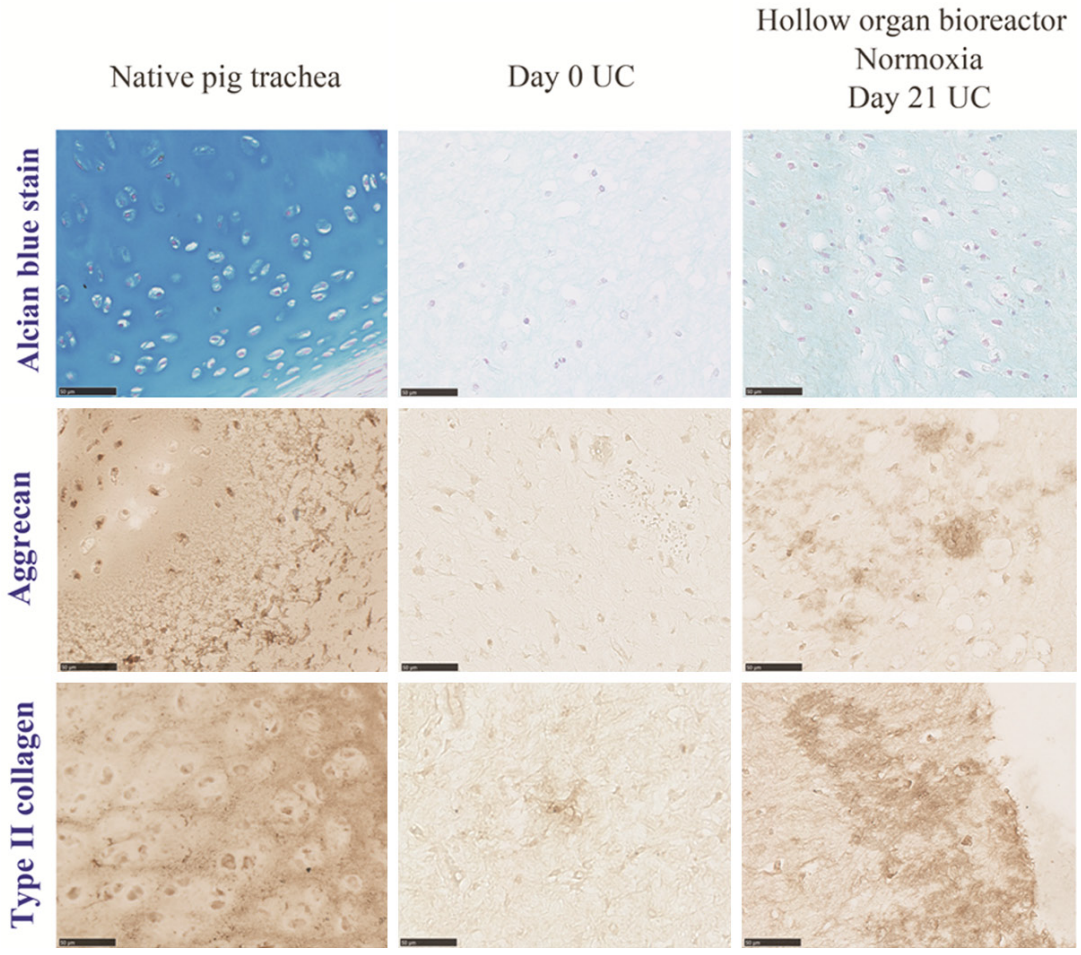

Native pig trachea
Day 0 UC
Hollow organ bioreactor
Normoxia
Day 21 UC
Alcian blue stain
Aggrecan
Type II collagen

Figure 5. The whole section Umbilical Cord (UC) cultured within a hollow organ bioreactor under nomoxia for 21 days, the expression of aggrecan/type II collagen analyzed by IHC, and GAGs with alcian blue stain compared with a native pig trachea and uncultured UC. 10 cm long umbilical cord tissues were cultured in a Chondrocyte Induced Medium (CIM) containing 10 ng/ml TGF-β1 under nomoxia for 21 days within a hollow organ bioreactor. The expression of aggrecan/type II collagen increased after being cultured with a CIM containing 10 ng/ml TGF-β1 under nomoxia for 21 days, but it seems there was moderate expression in the alcian blue stain. Blue color indicates GAGs expression. Brown color indicates Aggrecan/type II collagen expression. Magnification ×400X.

Table 1. Summary of the data on the extent of chondrogenesis in various conditions related to UC explants culture

Assays	Explants culture (N = 3)			
	0	10	20	40
	Hypoxia/Normoxia	Hypoxia/Normoxia	Hypoxia/Normoxia	Hypoxia/Normoxia
Chondrocytes* (Lacuna-like cell numbers)	+/+	++/++	++/++	++/++
Aggrecan	+/-	+++/+	+++/+	+++/+
Type II collagen	+/-	+++/+	++/+	+/+
Alcian blue	-/-	+/+	-/-	-/-
TUNEL assay (Apo-BrdU)	+++/+++	+++/+++	+++/+++	+++/+++

TGF-β1: Cultured with TGF-β1 0, 10, 20 or 40 ng/ml for 21 days (N = 3/ group). *: 1~10 chondrocytes (+); 11~20 chondrocytes (++). IHC/Alcian blue signal intensity: Strong (+++); Moderate (++); Weak (+); No detection (-). Apo-BrdU intensity: 0~5 apoptotic cells (-); 6~30 apoptotic cells (+); 31~55 apoptotic cells (++); 56-90 apoptotic cells (+++).

trol group) and uncultured UC. More apoptotic cells than tracheal cartilage control were found in all other groups. Regardless of hypoxia or normoxia conditions, apoptosis appeared similar in all TGF-β1 cultured-UC explants. These data suggest that UC explants cultured under miscellaneous conditions, including TGF-β1, hypoxia and different bioreactors, consistently undergo apoptosis.

Discussion

Our results show that there was more chondrocyte growth under hypoxia, as well as during the use of the rotating bioreactor when only in the presence of 10 ng/ml TGF-β1. Further assessments involving versatile stains have validated the nature of premature chondrocytes, where only phenotypes lacking GAG product formation appeared, similar to the embryo stage of chondrocytes. Up until now, under currently used differentiation protocols, stem cells have been unable to fully differentiate into mature and functional chondrocytes [18]. Literature reviews show that studies using MSCs for chondrogenesis effectuates only premature chondrocytes. No attempts have been successful in duplicating mature chondrocytes regeneration, like those which are using articular cartilage-based chondrocyte sources. Our current findings have involved only premature chondrocyte growth. Obvious phenotypes lacking mature GAGs secretion were found in cultured whole section UCs. The possible factors involved in this include an inherent amniotic membrane barrier restricting the penetration of nutrients, oxygen and metabolic waste, and the unprocessed UC containing intact Extracellular Matrix (ECM) without an extra compartment for outnumbered chondrocytes. These defects could be fixed in the future with improved designs, such as diffuse pin-hole punctures on the outer amniotic membrane, or having adequate enzyme digestion to create more pericellular rooms for better secretion of chondrocyte ECM. Previous researchers had used small volumes of UCs obtained from humans, cows, and pigs by means of aseptic caesarean incisions. Here, we used an unconventional approach to collect UCs via the use of uncomplicated surgeries, and were thus able to successfully harvest UCs at larger volumes during natural deliveries. The amniotic membrane posed an inherent barrier which was resistant to a disinfection solution used routinely during the aseptic procedure, while protecting the inner UC tissues which provide resources for MSC. The UC inherits an intact natural gel similar to synthetic hydrogel. A physiological concentration of merely 10 ng/ml TGF-β1 could well be enough to directly induce chondrogenesis in UC whole explants, despite the presence of the protective amniotic membrane and WJ with an intact ECM.

Table 2. Summary of the data compared with a native pig trachea and uncultured UC on the extent of chondrogenesis (by cell count, IHC and alcian blue stain) and apoptosis in a rotatory bioreactor under hypoxia of UC explants culture, and a hollow organ bioreactor under normoxia compared with a native pig trachea and uncultured UC

Assays \ Culture condition (N = 3)	Pig Native trachea	Day 0 UC	Explants culture Rotatory bioreactor (10 ng/ml TGF-β1) Hypoxia	Whole section culture Hollow organ bioreactor (10 ng/ml TGF-β1) Normoxia
Chondrocytes* (Lacuna-like cell numbers)	++++	+	++	++
Aggrecan	++++	+	++	+++
Type II collagen	++++	+	++	+++
Alcian blue	++++	-	-	++
TUNEL assay (Apo-BrdU)	-	+	+++	+++

Native trachea: Fresh listed porcine (6-month-old) trachea as base line (mature cartilage). Day 0 UC: Umbilical cord (UC) before culture (N = 3), + means discrete morphology of lacuna cell. Rotatory bioreactor (10 ng/ml TGF-β1): Cultured with 10 ng/ml TGF-β1 in rotatory bioreactor for 21 days under hypoxia (N = 3). *: 1~10 chondrocytes (+); 11~20 chondrocytes (++); 21~30 chondrocytes (+++); 31~40 chondrocytes (++++); >41 chondrocytes (+++++). IHC/Alcian blue signal intensity: Excellent (++++); Strong (+++); Moderate (++); Weak (+); No detection (-). Apo-BrdU intensity: 0~5 apoptotic cells (-); 6~30 apoptotic cells (+); 31~55 apoptotic cells (++); 56-90 apoptotic cells (+++).

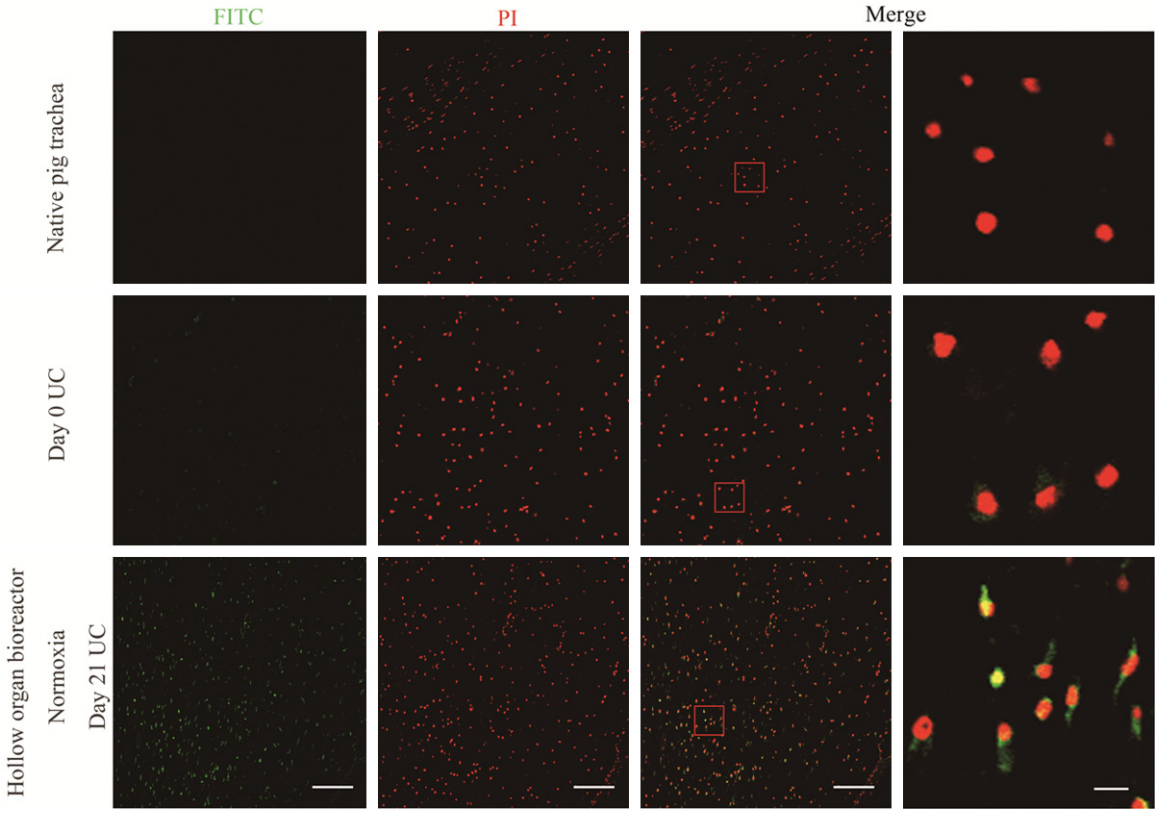


Figure 6. Apoptotic cells in the whole section Umbilical Cord (UC) cultured within a hollow organ bioreactor under nomoxia for 21 days compared with a native pig trachea and uncultured UC. 10 cm umbilical cord tissues were cultured in a Chondrocyte Induce Medium (CIM) containing 10 ng/ml TGF-β1 under nomoxia for 21 days within a hollow organ bioreactor. FITC: Green fluorescence labeled cells indicate apoptotic signal (Apo-BrdU); PI: Red fluorescence labeled total cells; Merge: Merging green and red fluorescence. Lines 1 to 3 (scale bar = 100 μm with magnification ×200X); the merged data of FITC/PI was the same area as the red box in line 3, and at high magnification in line 4 (scale bar = 10 μm).

Certain characteristics of chondrocytes grown in UCs included an uneven distribution over various sections with discrete lacuna-like morphology, along with the absence of safranin O and weak alcian blue stainings. Only the type II collagen and aggrecan positive IHC stainings could confirm the cell nature of chondrocytes. The typical UC chondrogenesis seen on explants and whole section cultures merely represent a primitive stage of chondrocyte formation. The type II collagen and aggrecan loomed simultaneously during the early stage of chondrogenesis, followed by positive reactions to alcian blue, and lastly to safranin O. Positive staining with safranin O was one of the measures which supported the mature chondrocytes phenotype with appropriate differentiation. The chondrocytes were likely in a premature state with no GAG expressions or positive reaction to safranin O staining, as well as having a weak alcian blue staining comparable to the native pig tracheal sections. The current data suggest that direct *in situ* chondrogenesis occurred in the UC explants, and were lacking these positive stainings despite definite positivity being noted in the pig native trachea. Still, these results remain to be improved in the future.

UC explants cultured in CIM containing TGF-β1 under normoxic or hypoxic conditions all produced lacuna-like cell morphology and aggrecan/type II collagen expressions, particularly in hypoxic environments. However, we found no significant differences in the results across the 20 and 40 ng/ml TGF-β1 settings. For the entire section of pig UC covering the tracheal scaffold (and equipped in a hollow organ bioreactor chosen CIM at 10 ng/ml TGF-β1 for a 21-day culture) under normoxia, lacuna-like cell formation and aggrecan/type II collagen expressions increased. Meanwhile, we discovered there were moderate levels of apoptotic cells seen in whole section UC cultures in the hollow organ bioreactor. Our concentration of 10 ng/ml TGF-β1 for chondrogenesis was much lower than that which Michael et al. had used for synthetic gel [19]. This accounts for the high levels of TGF-β1 necessary to help penetrate the gel for vivid chondrogenesis. Perhaps, we could have increased the concentration of TGF-β1 to >200 ng/ml, and/or utilized enzyme digestion with physical drilling on the amniotic membrane to facilitate the movement of nutrients to tissues which were located more centrally in order to enhance more numerous chondrogenesis. Our results from monitoring cell viability revealed that apoptosis had occurred during tissue culture. Although as previously reported, while explant cultures sustained long-standing MSC stemness and survival, the non-MSC cells required for vivid growth may not be well preserved, particularly those in hypoxic condition. Our findings suggest that whole section UCs can only provide excellent short term MSC survival in cultures in terms of coexisting apoptosis, when the duration of culture did not exceed a week. We recommend that for feasible tissue engineering, differentiated whole section UCs be initially cultured *in vitro* for one week without obvious apoptosis, prior to their being implanted in a living animal. The limitation of our study was having obscure stains in whole UC sections without an obvious comparison for native cartilage. Otherwise, use of a hollow organ bioreactor combined with hypoxia culture could not achieve a theoretically optimal culture due to lack of an adequate facility.

Even though this body of research has the undeniable merit of offering valuable insight into chondrocyte growth in readily available UCs, it had some limitations: (1) Hypoxia theoretically enhances chondrogenesis in UC; however, it coincidentally induced escalating apoptosis. This result appears odd to me. The optimal period for application on abundant chondrocyte growth in a hollow organ bioreactor needs to be further explored. (2) Heterogenesis of chondrocyte growth on UCs with an intact surface would produce biased data under an opportunistic slide specimen. We readily acknowledge that our research is exploratory and that there are problems with the statistical mode. Much work remains to be done; however, we anticipate that our study will generate important future findings in the fields surrounding promising chondrogenesis growth in UCs.

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Disclosure of conflict of interest

None.

Abbreviations

MSC-rich, multipotent stromal cell-rich; UCs, umbilical cords; TGF-β1, transforming growth factor-β1; H&E stain, hematoxylin and eosin stain; APO-BrdU TUNEL assay, Apoptosis-Bromodeoxyuridine (5-bromo-2'-deoxyuridine) Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling assay; ECM, extracellular matrix; GAGs, glycosaminoglycans; BM, bone marrow; WJ, wharton's jelly; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CIM, Chondrocyte induction medium; IHC, Immunohistochemistry.

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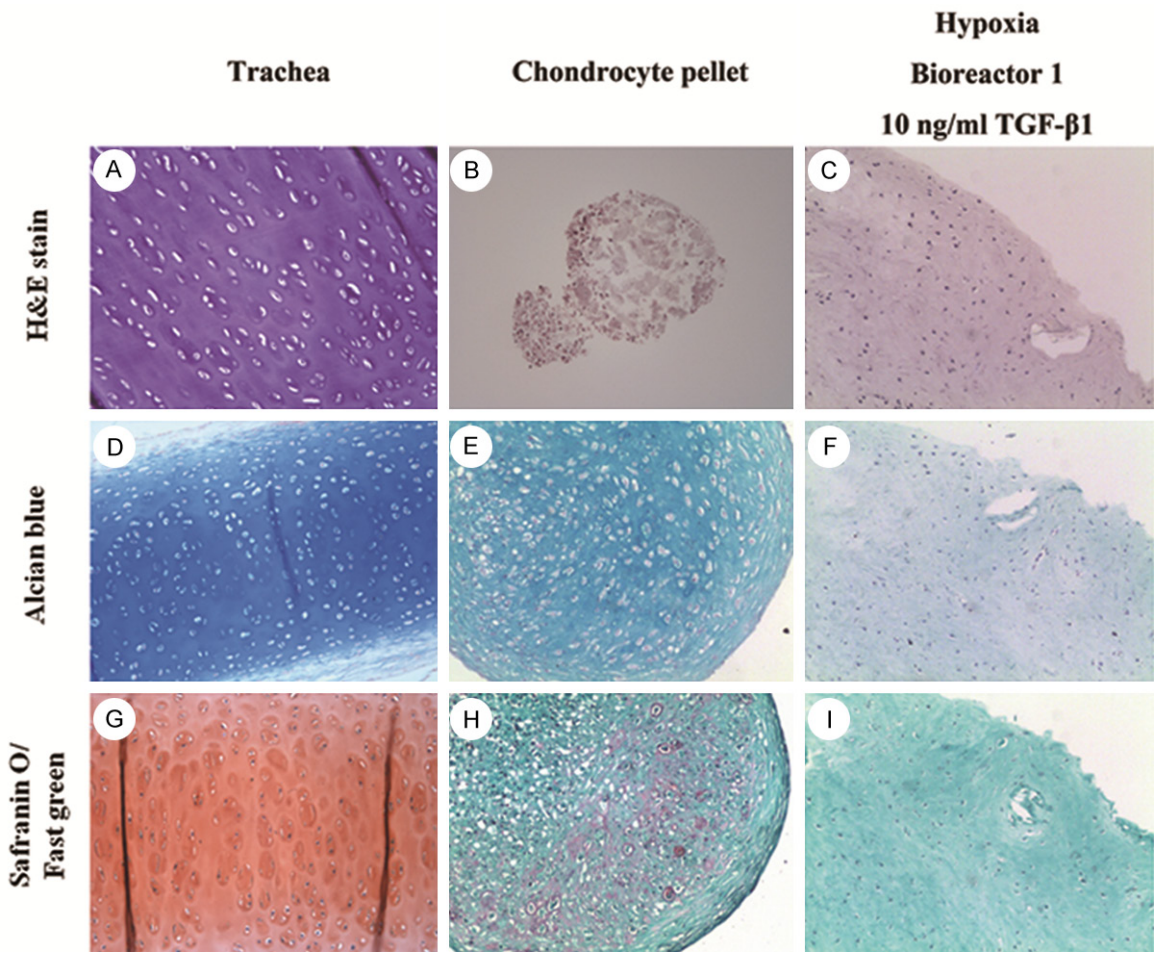
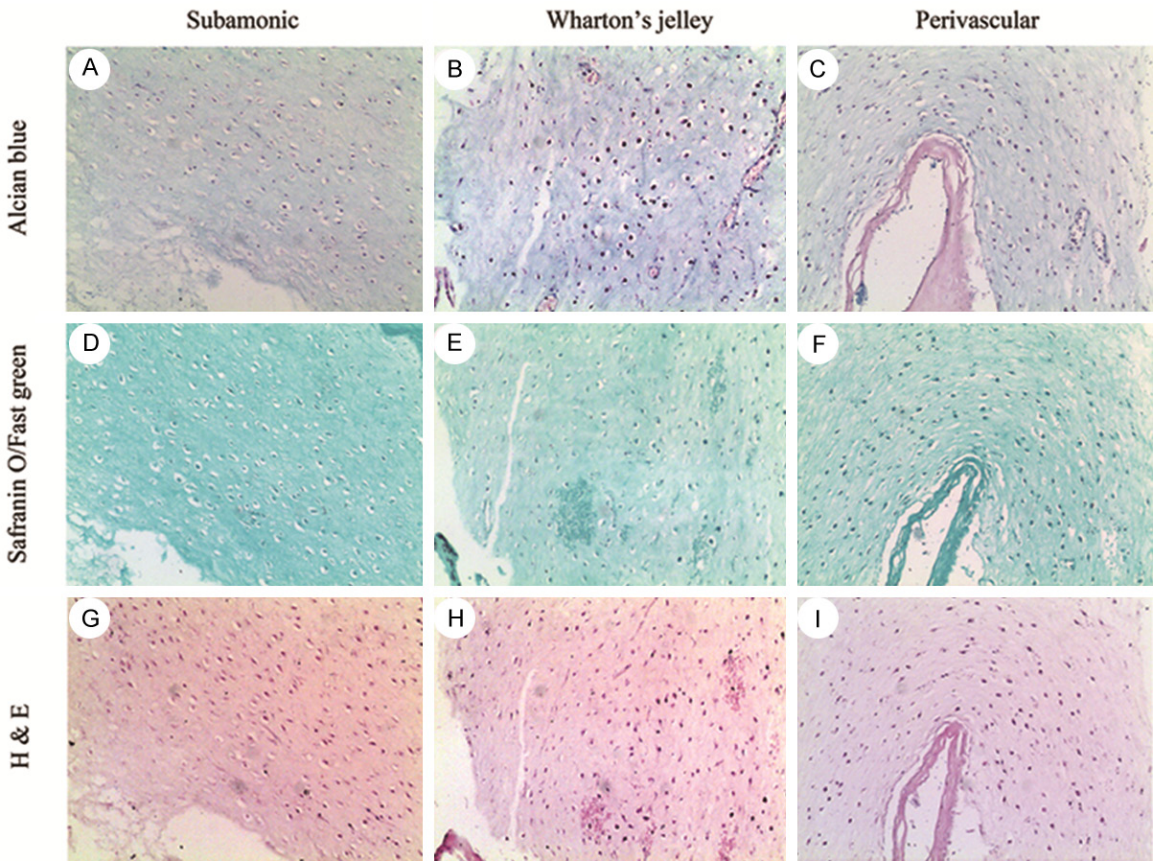
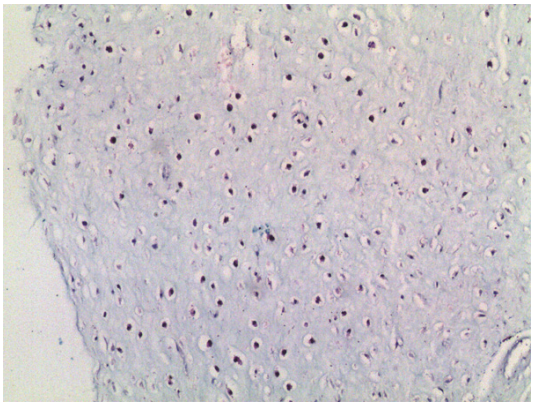


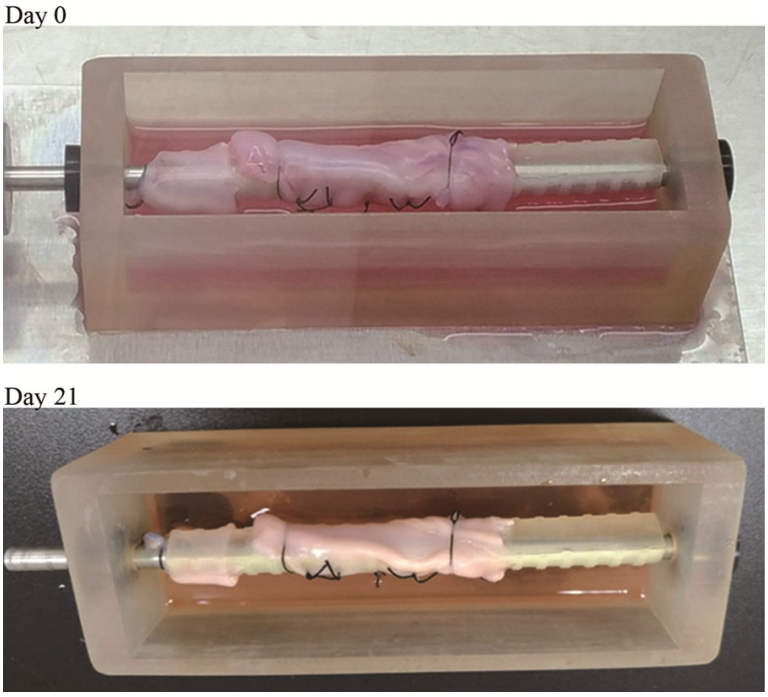
Supplementary Figure 1. Showing results of staining with H&E (A-C), alcian stain (D-F) or safranin O (G-I) in pig trachea, chondrocyte pellet and pig UC after 21 days culture. (A, D, E) Native pig trachea. (B, E, H) Dissociated WJ-MSCs of porcine UC with formed chondrocyte pellets. (C, F, I) Pig UCs cultured in CIM containing 10 ng/ml TGF-β1 in bioreactor-1 under hypoxia for 21 days. Magnification ×200X.



Supplementary Figure 2. Showing results of alcian blue (A-C), safranin O (D-F) and H&E (G-I) stains in subamonic, wharton's jelley and perivascular areas of porcine UC after 21 days culture. UCs were cultured in CIM containing 10 ng/ml TGF-β1 for 21 days under normoxia. Magnification ×200X.



Supplementary Figure 3. Premature chondrocyte growth was detected by alcian blue stain within WJ of cultured UC sections. Magnification ×200X.



Supplementary Figure 4. The whole section Umbilical Cord (UC) cultured within a hollow organ bioreactor under nomoxia for 0 and 21 days. The alteration in the mechanical indices after 21 days included shrinkage of wharton's jelley despite intact ammonic membrane with same elasticity as day 0.