BIOCELL
2022 46(9): 2089-2099

Tech Science Press

Wnt3a-induced ST2 decellularized matrix ornamented PCL scaffold for bone tissue engineering

Xiaofang WANG[1]; Xiaolin TU[1,2,*]; Yufei MA[1]; Jie CHEN[1]; Yang SONG[3]; Guangliang LIU[1]

[1] Laboratory of Skeletal Development and Regeneration, The Institute of Life Science, Chongqing Medical University, Chongqing, 400016, China
[2] Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN46202, USA
[3] Bioengineering Collage, Chongqing University, Chongqing, 400044, China

Key words: Wnt3a, Decellularized matrix, Bone marrow stromal cells, Osteogenic differentiation, Bone tissue engineering

Abstract: The limited bioactivity of scaffold materials is an important factor that restricts the development of bone tissue engineering. Wnt3a activates the classic Wnt/β-catenin signaling pathway which effects bone growth and development by the accumulation of β-catenin in the nucleus. In this study, we fabricated 3D printed PCL scaffold with Wnt3a-induced murine bone marrow-derived stromal cell line ST2 decellularized matrix (Wnt3a-ST2-dCM-PCL) and ST2 decellularized matrix (ST2-dCM-PCL) by freeze-thaw cycle and DNase decellularization treatment which efficiently decellularized >90% DNA while preserved most protein. Compared to ST2-dCM-PCL, Wnt3a-ST2-dCM-PCL significantly enhanced newly-seeded ST2 proliferation, osteogenic differentiation and upregulated osteogenic marker genes alkaline phosphatase (*Alp*), *Runx2*, type I collagen (*Col 1*) and osteocalcin (*Ocn*) mRNA expression. After 14 days of osteogenic induction, Wnt3a-ST2-dCM-PCL promoted ST2 mineralization. These results demonstrated that Wnt3a-induced ST2 decellularized matrix improve scaffold materials' osteoinductivity and osteoconductivity.

Introduction

The regeneration of large bone defects is a major challenge in the field of orthopedics (Schmidt, 2021). Autologous bone is the gold standard for repairing such defects. However, the morbidity of the donor site and the size of the graft limit its clinical application (Al-Abedalla *et al.*, 2015). Bone grafts of the same species and xenotypes have been used clinically. But they can cause immunological rejection which lead to migration failure. What's more, they have differences in bone regeneration for the gender, age, race, and living habits of the donors, which restricted their clinical practices (Tournier *et al.*, 2021; Visscher *et al.*, 2016). With the continuous development of tissue engineering technology, artificial bones can be produced in large quantities (Almubarak *et al.*, 2016). 3D printing technology is favored for personalized manufacturing scaffolds (Battafarano *et al.*, 2021; Wu *et al.*, 2019). And scaffold materials play an important role in the construction of artificial bones. The ideal scaffold material should have similar components to natural bone, provide similar mechanical strength and biological microenvironment, which promote adhesion, growth, proliferation, migration and differentiation of cells on the scaffold (Chae *et al.*, 2021; Nikolova and Chavali, 2019). However, the current scaffold materials cannot provide complex cell signals in natural bone microenvironment, and bone repair effect needs further enhanced (Long *et al.*, 2015; Shang *et al.*, 2021). Living cells are printed to fabricate bone repair scaffolds in bone tissue engineering to improve scaffold materials' bioactivity, which will cause ethical issues, and the transformation to clinical trials is still challenging (Bose *et al.*, 2013). Therefore, the development of scaffold materials with good osteogenesis and mechanical properties has become a research hotspot in bone tissue engineering.

Decellularized matrix (dCM) removes immunogenic substances such as DNA from cells, while retains the precise and orderly network structure composed of proteins and polysaccharides (Bracaglia and Fisher, 2015), and its three-dimensional microstructure can provide the body closest to cell growth. The dCM is rich in various active molecules to provide the basis for various cell activities (Aamodt and Grainger, 2016). Decellularized matrix (dCM) has been widely used as scaffold materials for regenerative medicine due to its natural properties, low immune repulsion and excellent biocompatibility. Demineralized bone matrix (DBM), a kind of allograft material, has been used to enhance joint fusion and spinal fusion in clinic orthopaedic surgery for its similar

*Address correspondence to: Xiaolin Tu, xtu@cqmu.edu.cn
Received: 18 November 2021; Accepted: 03 January 2022

Doi: 10.32604/biocell.2022.020069

composition, structure and function to autologous bone (Drosos *et al.*, 2007). However, the osteogenic activity of commercially available DBM depends on specific donor characteristics, such as age, sex and lifestyle and its preparation method. In addition, several commercial decellularized matrix have received FDA approval for use in humans, including dermal tissue, pig heart valve and porcine bladder. Decellularized scaffolds made from porcine small intestinal submucosa (SIS) have been used in orthopedic surgery to repair rotator cuff in preclinical trials. Although tissue-derived biological scaffolds are commonly used to repair non-homologous anatomical sites, studies of tissue engineering in skeletal muscle suggested that biological scaffolds from homologous tissues at specific sites may be more suitable for constructive tissue remodeling than non-site-specific scaffolds (Zhang *et al.*, 2022). Decellularized bone effectively promotes bone regeneration, but its source is limited and cannot meet clinical needs (Cheng *et al.*, 2014). Cell-derived dCM has attracted much attention due to its wide range of sources (Parmaksiz *et al.*, 2020). Studies have found that dCM of fibroblasts and endothelial cells promoted angiogenesis (Fu *et al.*, 2018). Chondrocyte dCM promotes cartilage repair (Pei and He, 2012). Mesenchymal stem cells (marrow mesenchymal stem cells, MSCs) dCM-modified titanium scaffold upregulated the expression of osteogenic genes of mesenchymal stem cells (Benders *et al.*, 2013). Osteoblast (MC3T3-E1) dCM-modified calcined bone accelerated the repair of rabbit radius defects (Ma *et al.*, 2017). A large number of studies have proved that the cell-derived decellularized matrix has great potential as a tissue engineering repair material.

The Wnt/β-catenin signaling pathway plays an important role in the growth and development of bone (Benoit, 2014). The activated classic Wnt signaling pathway promotes the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by upregulating osteogenic differentiation related genes (Zhong *et al.*, 2012). As the protein of classic Wnt signaling pathway, Wnt3a protein is widely involved in regulating cell biological behavior (Boland *et al.*, 2004). The previous study of our group found that Wnt3a protein promoted osteogenic differentiation of BMSCs (Tu *et al.*, 2007), but Wnt protein is unstable *in vivo* (Boland *et al.*, 2004). Therefore, in this study, we modified 3D printed PCL scaffold with decellularized matrix from Wnt3a-induced murine bone marrow-derived stromal cell line ST2 (Minetaro Ogawa *et al.*, 1988) which is widely used in osteogenic differentiation for its osteogenic potency, easy to obtain and culture *in vitro* (Li *et al.*, 2019a; Yuan *et al.*, 2021). We evaluated Wnt3a-induced ST2 dCM ornamented PCL scaffold on ST2 activity, proliferation, osteogenesis and mineralization. The results proved Wnt3a-induced ST2 dCM endow the scaffold material with biological activity and is a new potential bone repair scaffold materials for bone tissue engineering.

Materials and Methods

Reagents

Polycaprolactone (PCL) and cetylpyridinium chloride were purchased from Aladdin Reagent Co., Ltd., Shanghai, China. Fetal Bovine Serum and α-Minimum Essential Medium were purchased from Biological Industries Israel Beit Haemek Ltd, Beit Haemek, Israel. Reverse transcription kit, real-time fluorescent quantitative PCR kit and Trizol were purchased from Accurate Biotechnology Co., Ltd., Hunan, China. Pancreatin, penicillin-streptomycin, RIPA Lysis Solution, BCIP-NBT Alkaline Phosphatase Color Development Kit, Alkaline Phosphatase Assay Kit, BCA Protein Assay Kit and hemotoxylin-eosin staining were purchased from Beyotime Institute of Biotechnology, Shanghai, China. β-catenin antibody was purchased from Wanlei Institute of Biotechnology, Shenyang, China. Hochest 33258 nuclear staining solution and DNaseI were purchased from Solarbio Institute of Biotechnology, Beijing, China. Quant-iT^{TM} PicoGreen® dsDNA Reagent Kit and LIVE-DEAD^{TM} cell viability Kit were purchased from Invitrogen, USA. Cell Counting Kit-8 was purchased from Dojindo Laboratories (Japan).

Cell culture and collection of Wnt3a conditioned medium

Wnt3a condition medium was collected as previous study (Tu *et al.*, 2007). In brief, murine bone marrow-derived stromal cell line ST2 and Wnt3a cells were cultured in basal medium (α-MEM medium containing 10% FBS and 1% penicillin-streptomycin). Then ST2 was cultured in Wnt3a conditioned medium to basal medium at ratio of 0, 1:2, 1:4, and 1:8 for 3 days to detect the appropriate ratio.

RT-qPCR analysis

RT-qPCR was performed as previous study (Tu *et al.*, 2007). Osteogenic marker genes expression of *Alp, Runx2, Osx, Ocn* and *β-catenin* and Wnt target genes *Smad6*, *Axin2*, *BMP4*, *Lef1* mRNA was detected. The relative gene expression level was analyzed by $2^{-\Delta Ct}$ method. The genes and corresponding primers are listed in Table 1. Alkaline phosphatase staining was used to detect the level of osteogenic differentiation. β-catenin immunofluorescence staining was used to detect β-catenin entering the nucleus.

3D-printed PCL scaffold

Scaffolds were printed by polycaprolactone (PCL) using a 3D bioprinter according to a predesigned 3D structure model and then cut into 5 × 5 × 1 mm scaffolds. The scaffolds were immersed in 75% ethanol for 1 h, then immersed in sterile PBS 3 times under UV light before cell seeding.

Preparation of PCL scaffolds ornamented with decellularized matrix

Decellularized matrix ornamented PCL scaffolds were prepared as a previous study (Pati *et al.*, 2015). In short, PCL scaffolds were seeded with 1 × 10*5* ST2 and incubated in incubator 2 h allow cells attach to scaffolds. And then basal medium and Wnt3a conditioned medium to α-MEM medium 1:4 were added respectively. After 3 days, two group scaffolds were decellularized by three freeze-thaw (F-T) cycles in liquid nitrogen and 37°C water bath (10 min each) respectively. Then the scaffolds were incubated in 1 mL DNase I solution (0.2 mg/mL) in an incubator for an hour. DNase is an enzymatic decellularization agent, used for breaking down DNA fragments and removal of the nucleotides lysis. There are no reported adverse effects of DNase on matrix (Pati *et al.*, 2015). The goal of the

TABLE 1

Primers for real-time PCR

Gene	Forward sequence (5'-3')	Reverse sequence (3'-5')
Alp	TATGTCTGGAACCGCACTGAA	CACTAGCAAGAAGAAGCCTTT
Runx2	ATCCAGCCACCTTCACTTACA	GGGACCATTGGGAACTGATAG
Ocn	AACGGTGGTGCCATAGATGC	AGGACCCTCTCTCTGCTCAC
Col 1	CAGGCTGGTGTGATGGGATT	CCAAGGTCTCCAGGAACACC
Smad6	AAGATGCTGAAGCCGTTGGT	CGAACTCCAGTATCTCCGCTTT
Axin2	TGCAGGAGGCGGTACAGTTC	GCTGGAAGTGGTAAAGCAGCTT
BMP4	GAGGAGTTTCCATCACGAAGA	GCTCTGCCGAGGAGATCA
Lef1	TACCCCAGCCAGTGTCAACA	TCCATGATAGGCTTTGATGACTTTC
GAPDH	AACTCCCATTCTTCCACCTTT	CTCTTGCTCTCAGTATCCTTG

decellularization process was to maximize removal of cellular debris while minimizing the disruption of CM components.

Evaluation of decellularization effect

Before and after decellularization, scaffolds were fixed with 2.5% glutaraldehyde in PBS for 1 h, then washed with PBS and dehydrated successively in 70%, 90%, 95%, and 100% ethanol for 15 min each. The fixed samples were vacuum dried, sputter-coated with platinum, and examined under a scanning electron microscope operated at 10 kV.

To verify the decellularized effect, DNA and total protein were quantified before and after decellularization. The total DNA was quantified by Quant-iT^{TM} PicoGreen$^{®}$ dsDNA Reagent Kits. Cell nuclei stained with Hoechst 33258 was also used to determine whether the decellularization process was completed. Two group scaffolds were extracted with 300 μL RIPA lysis buffer in respectively. The protein content accumulating on scaffolds was tested by BCA Protein Assay Kit as the direction. Three samples were tested for each condition.

Effect of dCM ornamented PCL scaffolds on ST2 viability and proliferation

Cell viability and proliferation were detected as a previous study (Yu *et al*., 2020). ST2 were seeded on PCL and dCM ornamented PCL scaffolds to study the effect of dCM on cell viability, proliferation, and differentiation. Cell-seeded scaffolds were incubated 2 h to allow cells attach to scaffolds, then basal medium was added. Cell viability was assessed by live-dead cell viability assay 24 h later and live-dead rate was calculated by ImageJ. Cell proliferation was performed on days 1, 4, and 7 by CCK-8 assay. 10% CCK-8 solution was added to each sample and incubated at 37°C for 1 h, then measured absorbance at a wavelength of 450 nm using a microplate reader (Biotek Instruments, USA).

Effect of dCM ornamented PCL scaffolds on ST2 osteogenesis

For osteogenic differentiation, 1×10^5 ST2 were seeded on dCM ornamented PCL scaffolds for 7 and 14 days respectively. Then two group scaffolds were stained for alkaline phosphatase (ALP) by BCIP-NBT Alkaline Phosphatase Color Development Kit. Quantitative real-time PCR (qRT-PCR) was performed to detect four osteogenic marker genes expression. The study was performed in triplicate.

For assessing mineralization, the cell proliferating medium was replaced by osteogenic medium after 7 days and then cultured for 14 days. Alizarin red S staining was performed to evaluate the calcium deposition of different groups. The scaffolds were stained by alizarin red stain at room temperature for 10 min and the calcium nodules were examined under microscope after washing five times with deionized water. For quantitative analysis, the stained samples were air-dried and immersed in 10% cetylpyridinium chloride for 20 min to elute the stain which was prepared according to a previous report (Chen *et al*., 2020). The absorbance of the elution solution was measured at 562 nm.

Results

The appropriate ratio of Wnt3a conditioned medium

ST2 cells were cultured in Wnt3a conditioned medium to basal medium at ratio of 0, 1:2, 1:4, 1:8 for 3 days. Alkaline phosphatase staining, Alp activity and qPCR results showed that ST2 osteogenic differentiation and osteogenic marker genes *Alp, Runx2, Ocn, and Col 1* mRNA expression were significantly strongest in Wnt3a conditioned medium to basal medium at the ratio of 1:4 group among the four groups ($P < 0.05$) (Figs. 1A–1C). In the Wnt3a conditioned medium to basal medium at ratio of 1:4 group, a large amount of β-catenin can be seen into the nucleus, and there were >50% β-catenin positive cells (Figs. 2A and 2B). QPCR results showed that *β-catenin* and Wnt signaling target genes *Smad6, Axin2, BMP4, Lef1* mRNA expression were significantly higher than control (Fig. 2c). These results indicated that Wnt3a conditioned medium to basal medium at the ratio of 1:4 has best osteogenic effect, so the following experiments use this ratio of medium to induce ST2.

Characterization of dCM ornamented PCL scaffold

SEM micrographs showed the surface morphology of the scaffolds before and after decellularization. Cells proliferated well on the scaffolds. After 3 days incubation, the scaffold surface was almost entirely covered by proliferating cells and their synthesized matrix (Fig. 3B). After decellularization, residual aggregations and layers of the cellular matrix could be observed on the scaffolds (Fig. 3e).

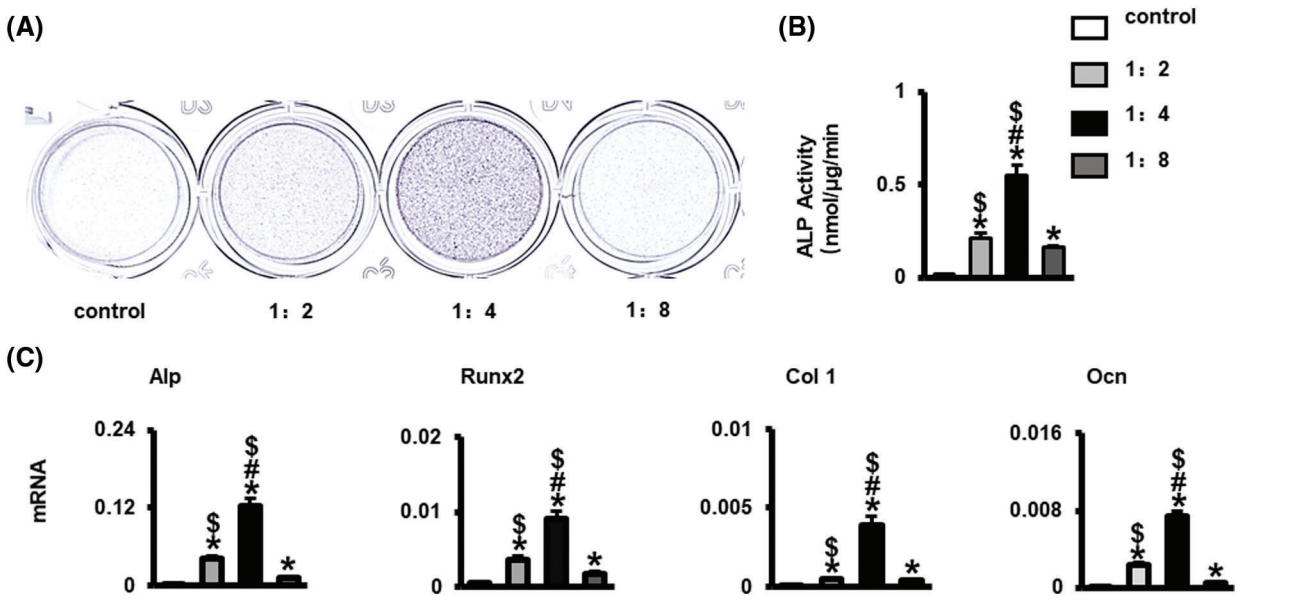

FIGURE 1. Wnt3a conditioned medium to basal medium at the ratio of 1:4 promoted ST2 osteogenesis. (A, B) AP staining and AP activity of ST2 osteogenic differentiation after culturing in Wnt3a conditioned medium to basal medium at the ratio of 0,1:2,1:4,1:8, respectively. (C) Gene expressions of *Alp, Runx2, Col 1 and Ocn* after 3 days culturing. Data were expressed as mean ± SD. **P* < 0.05 *vs.* control; #*P* < 0.05 *vs.* 1:2 group; $*P* < 0.05 *vs.* 1:8 group by one way ANOVA. Control means the group without Wnt3a conditioned medium. 1:2, 1:4 and 1:8 mean the ratio of Wnt3a conditioned medium to basal medium is 1:2, 1:4 and 1:8 group, respectively.

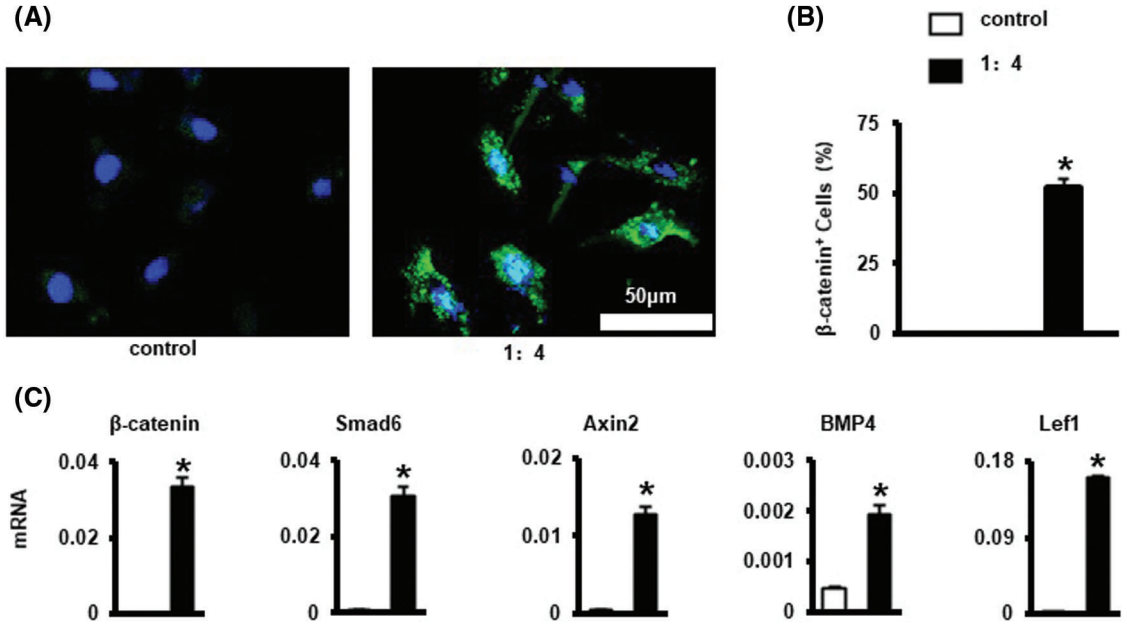

FIGURE 2. Wnt3a conditioned medium to basal medium at the ratio of 1:4 promoted β-catenin into the nucleus of ST2. (A) Immunofluorescence staining of β-catenin into the nucleus, the green fluorescence is β-catenin, and the blue fluorescence DAPI is the nucleus. Scale bars 50 μm. (B) The number of β-catenin staining positive cells. (C) Gene expressions of β-catenin and Wnt signaling target genes Smad6, Axin2, BMP4, and Lef1. Data were expressed as mean ± SD. **P* < 0.05 *vs.* control by one way ANOVA. Control means the group without Wnt3a conditioned medium; 1:4 means the ratio of Wnt3a conditioned medium to basal medium is 1:4.

Even after decellularization, the CM was well attached to the polymer surface (Fig. 3C). The CM attached to the surface of the scaffolds can provide an anchoring site for the cells and can influence cellular attachment and proliferation.

Evaluation of the effect of decellularization

To obtain dCM ornamented PCL scaffolds, cell-seeded 3D printed PCL scaffolds were cultured for 3 d, then decellularized. Hochest 33258 staining showed only a small amount of blue fluorescent staining of cell nuclei can be seen on the decellularized scaffold (Fig. 4A). The DNA and decellularized matrix proteins of Wnt3a-ST2-PCL group before and after decellularization were higher than those of ST2-PCL group (Figs. 4A–4C). DNA quantification showed that more than 90% of DNA components were removed in both groups (Fig. 4B), and the total protein quantification results showed that more than 60% of cellular matrix proteins were retained on the decellularized matrix modified scaffold (Fig. 4C).

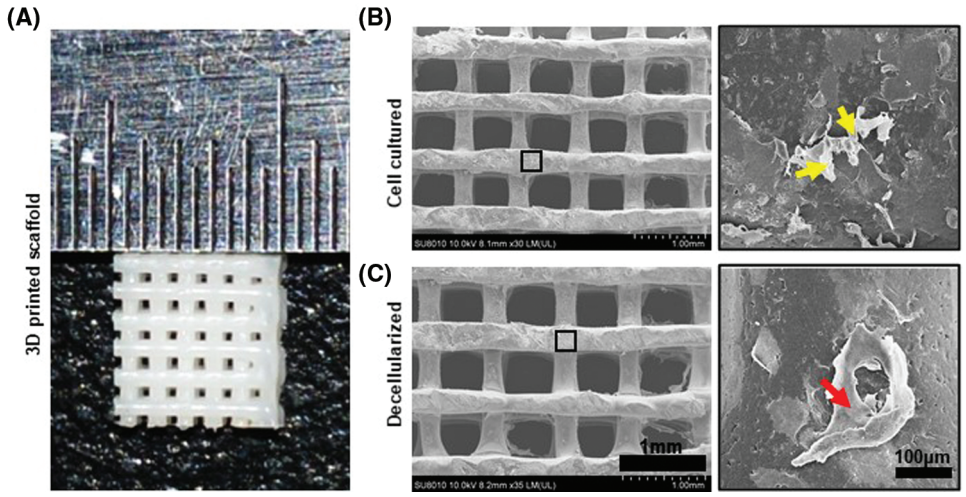

FIGURE 3. Characterization of dCM ornamented 3D printed PCL scaffold. (A) The scaffold is a 5 × 5 × 1 mm cube. (B) SEM images of the scaffolds before decellularization. Cells were marked with yellow arrow. The scaffold has a line width of 300 μm and pore size of 400 μm. Scale bars 1 mm. (C) SEM images of the scaffolds after decellularization. DCM was marked with red arrow. There was a large amount of cellular matrix attached to the scaffold, and almost no cell morphology and cell debris.

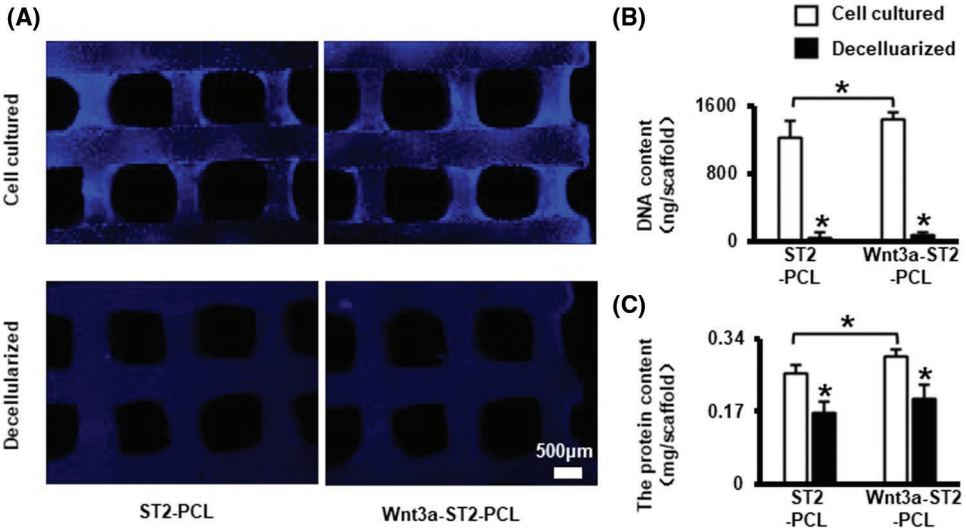

FIGURE 4. Freeze/thaw cycle and DNase decellularization removed >90% DNA and preserved >60% matrix. (A) Hochest 33258 staining for cell nucleus before and after decellularization. Scale bars 500 μm. (B, C) DNA and total protein quantitation of the scaffolds before and after decellularization. Data were expressed as mean ± SD. *$P < 0.05$ *vs.* cell cultured by one way ANOVA. ST2-PCL means PCL scaffold inoculated with ST2 cultured in basal medium; Wnt3a-ST2-PCL means the PCL scaffold inoculated with ST2 cultured in Wnt3a conditioned medium and basal medium at ratio of 1:4.

Effect of dCM ornamented PCL scaffolds on cell viability and proliferation

To evaluate the effects of dCM ornamented PCL scaffolds on cell viability, ST2 were cultured on PCL, ST2-dCM-PCL and Wnt3a-ST2-dCM-PCL scaffolds for 24 h and characterized by live-dead staining (Fig. 5A). <10% dead cells were seen in all three groups by ImageJ analysis (Fig. 5B), indicating decent biocompatibility of these scaffolds. CCK-8 tests at 1, 4, and 7 d showed that ST2 proliferated with the extension of the culture time in 3 groups (Fig. 5C), but proliferated faster on the scaffold modified with decellularized matrix, and the Wnt3a-ST2-dCM-PCL scaffold promoted ST2 proliferation significantly higher than the other two groups ($P < 0.05$) (Fig. 6B).

In vitro osteogenic differentiation of ST2 on dCM ornamented PCL scaffolds

After ST2 cultured on scaffolds 7 and 14 days in basal medium, AP staining presented highest on the Wnt3a-ST2-dCM-PCL scaffolds, ST2-dCM-PCL followed, and the pure PCL scaffold stained the lightest (Fig. 6A). *In vitro* osteogenic gene expression analysis clearly indicated that ST2 cultured in Wnt3a-ST2-dCM-PCL scaffolds showed significantly higher expression of *Alp, Runx2, Col 1, and Ocn* genes after 7 and 14 d than ST2-dCM-PCL and pure PCL scaffolds (Fig. 6B). These results implied that Wnt3a-ST2-dCM-PCL promoted early osteogenic differentiation of ST2 *in vitro*, possibly because Wnt3a-ST2-dCM produced several bioactive factors that stimulated osteogenic differentiation.

The effect of decellularized matrix ornamented PCL scaffolds on mineralization

In order to further evaluate the effect of dCM on ST2 mineralization, alizarin red staining was performed 14 days after osteogenic induction (Fig. 7A). Wnt3a-ST2-dCM-PCL had the most mineralized nodules. Quantitative analysis also confirmed the result of Alizarin Red staining (Fig. 7B). These results indicate that the Wnt3a-ST2-dCM-PCL scaffold has a better ability to induce ST2 mineralization *in vitro* than the other two groups.

Discussion

In recent decades, while the research of bone tissue engineering has made rapid progress, the comprehensive repair of large segment bone defects is still challenging, and effective therapies are constantly being explored (Al-Abedalla *et al.*, 2015). Decellularized matrix (dCM)

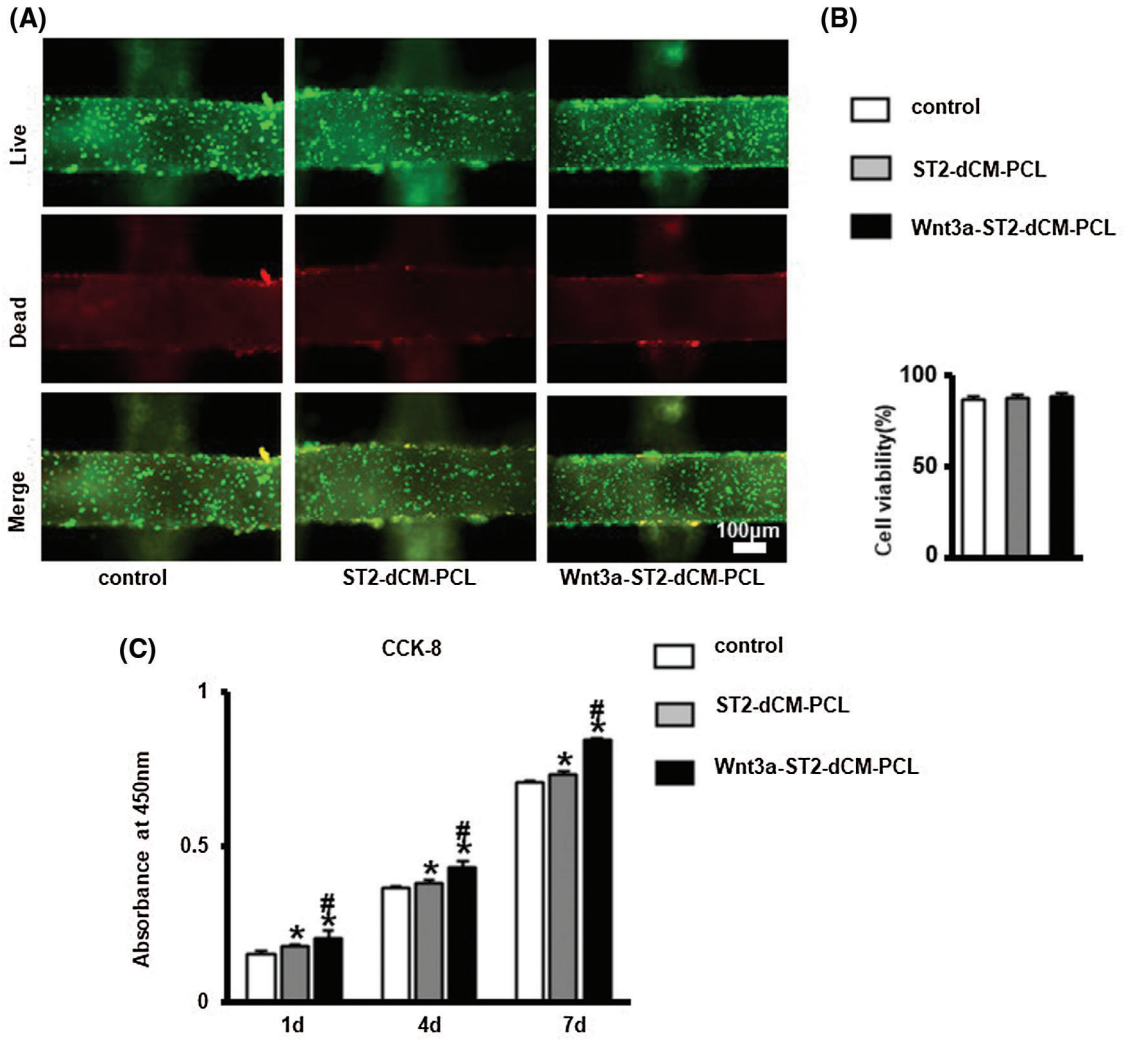

FIGURE 5. DCM ornamented scaffolds were biocompatible and Wnt3a-introduced ST2 dCM ornamented scaffolds promoted ST2 proliferation. (A) Live-Dead staining to analyze the viability of ST2 on the dCM ornamented scaffolds for 24 h, live cell (green) and dead cells (red). Scale bars 100 μm. (B) ImageJ analysis of the vitality of ST2 on dCM ornamented scaffolds. (C) CCK-8 analysis the proliferation of ST2 on dCM and PCL scaffolds at day 1, 4, 7. dCM, decellularized cellular matrix; Control, PCL scaffold; ST2-dCM-PCL means ST2 decellularized matrix ornamented PCL scaffold; Wnt3a-ST2-dCM-PCL means Wnt3a-induced ST2 decellularized matrix ornamented PCL scaffold.

composed of a complex network of proteins, proteoglycans, growth factors and other constituents (Mansour *et al.*, 2017). It is an ideal candidate for tissue engineering and regenerative medicine because most cells are in close contact with neighboring dCM environment that provides both biophysical and biochemical signals essential for cell adhesion, migration, and differentiation (Garcia and Martin, 2019). In addition, decellularized matrix can influence cell fate by directly binding to cell surface receptors. For example, decellularized matrix components such as type I collagen, fibronectin, and laminin bind to integrin receptors to activate pathways associated with osteogenic differentiation (Lin *et al.*, 2020). What's more, scaffold geometry, mechanical properties and surface morphology can affect cell behavior (Ballini *et al.*, 2017). We have ornamented scaffold with Wnt3a induced ST2-dCM to improve scaffold's bioactivity in our study. And dCM from human adipose stem cells significantly promoted the proliferation of bone marrow stromal cells (BMSCs), alkaline phosphatase (ALP) activity, calcium salt deposition, osteogenic gene markers and protein expression (Wei *et al.*, 2017). Decellularized matrix ornamented scaffold with BMP-2 can significantly promote BMSCs osteogenic differentiation and bone regeneration (Kim *et al.*, 2015). Pati' group constructed a dCM modified material scaffold from human inferior turbinate derived mesenchymal stem cells to simulate the bone microenvironment. The scaffold promoted osteoblast differentiation of newly implanted hTMSC and promoted bone formation at ectopic and *in situ* defects in rats by upregulating osteogenic marker gene expression and increasing calcium deposition (Pati *et al.*, 2015). Therefore, decellularized matrix will improve the properties of material scaffolds and has great potential in regenerative therapy. Recently, decellularization of tissue or organ has been widely used to obtain native enviromental mimicking scaffolds to modulate stem cell function (Silva *et al.*, 2020). In addition, combining dCM with biomaterials not only produced constructs with structural components and sustainable mechanical properties, but also growth factors and regulatory proteins that were conducive to tissue

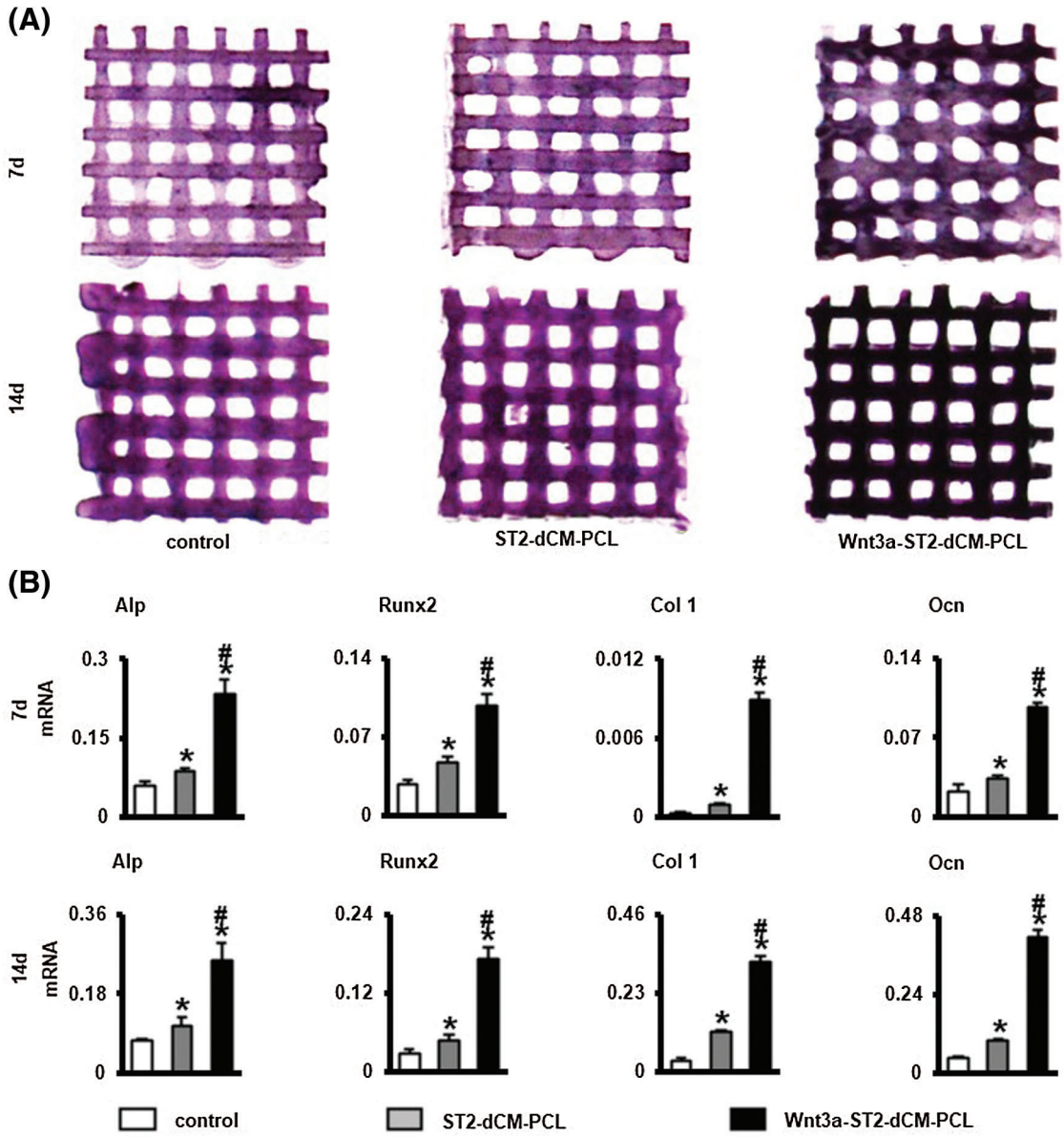

FIGURE 6. Wnt3a-introduced ST2 dCM ornamented scaffolds promoted ST2 osteogenic differentiation. (A, B) AP staining and AP activity assays of ST2 on dCM ornamented scaffolds after culturing in basal medium 7 and 14 d, respectively. (c) Gene expressions of *Alp, Runx2, Col 1* and *Ocn* after 7 and 14 d of culturing. dCM, decellularized matrix. Control, PCL scaffold; ST2-dCM-PCL means ST2 decellularized matrix ornamented PCL scaffold; Wnt3a-ST2-dCM-PCL means Wnt3a-induced ST2 decellularized matrix ornamented PCL scaffold. Data were expressed as mean± SD. **P* < 0.05 *vs.* control; . #*P* < 0.05 *vs.* ST2-dCM-PCL by one way ANOVA.

regeneration (Pei and He, 2012; Wang *et al.*, 2020). Furthermore, the combination of cell-derived dCM cultured *in vitro* with biomaterials allows the use of patient-specific cells to form a hybrid scaffold to eliminate donor site morbidity and anti-host immune response (Garcia and Martin, 2019). Cell-derived dCM generated by *in vitro* culturing has been considered a promising method among many kinds of decellularized matrix.

Wnt/β-catenin is one of the five major signal pathways of bone development. It participates in the entire osteogenic differentiation process. The activation of the classic Wnt/β-catenin signal promotes bone regeneration by promoting stem cell proliferation, osteogenic differentiation, and self-renewal (Zhong *et al.*, 2012). Wnt3a is a member of the Wnt protein family and is the main agonist protein of classic Wnt/β-catenin signaling. A large number of studies have found that Wnt3a can promote osteogenic differentiation (Tu *et al.*, 2007), but there are studies found that high-dose Wnt3a slows down the occurrence of osteogenesis differentiated stem cell matrix calcification progresses and inhibits osteogenesis. It can be seen that the regulation effect of Wnt3a on the osteogenic differentiation of stem cells is related to the dose of Wnt3a (Tu *et al.*, 2007). Studies have confirmed that Wnt3a promotes the osteogenesis of bone grafts (Boland *et al.*, 2004), but Wnt3a protein is not stable in the body and can cause adverse reactions in the body. Studies have found that reprogramming cells by small molecule compounds, exogenous transcription factors and biophysical cues regulated cell fate (Song *et al.*, 2021). Therefore, we constructed a Wnt3a-induced ST2 decellularized matrix modified scaffold for bone defect repair. Our previous studies have confirmed that Wnt3a cells secrete biologically active

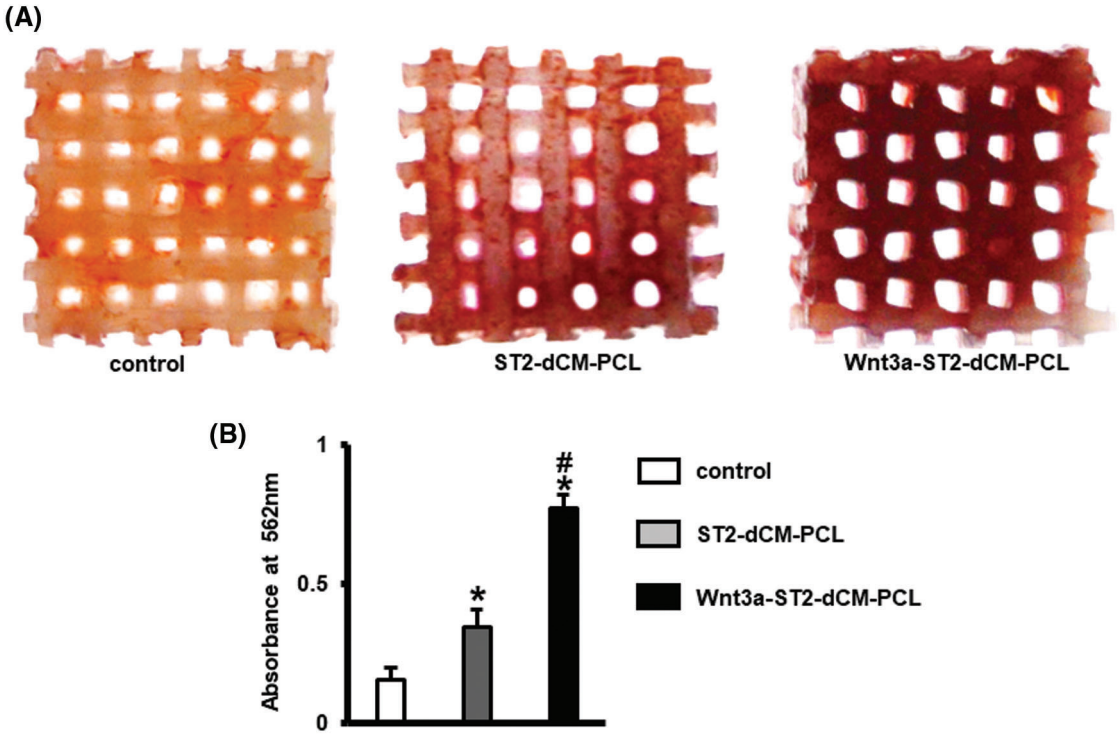

FIGURE 7. Wnt3a-introduced ST2 dCM ornamented scaffolds promoted ST2 mineralization. (A) Alizarin red S stained calcium mineral nodules deposited by ST2 after incubation for 14 d in osteogenesis induction medium on control, ST2-PCL, Wnt3a-ST2-PCL scaffolds respectively. (B) Alizarin Red activity were measured at day 21. dCM, decellularized matrix; Control, PCL scaffold ST2-dCM-PCL means ST2 decellularized matrix ornamented PCL scaffold; Wnt3a-ST2-dCM-PCL means Wnt3a-induced ST2 decellularized matrix ornamented PCL scaffold. Data were expressed as mean± SD. *$P < 0.05$ *vs.* control; . #$P < 0.05$ *vs.* ST2-dCM-PCL by one way ANOVA.

Wnt3a protein, which is a great cell source for Wnt3a conditioned medium (Tu *et al.*, 2007). Here, we screened the best ratio of Wnt3a conditioned medium to basal medium to promote ST2 osteogenic differentiation. Cellular matrix contains a variety of biologically active growth factors, which are increasingly used in tissue engineering (Mansour *et al.*, 2017). However, its poor mechanical properties limited its application. Scaffold materials can be designed to provide mechanical cues and regulate the epigenetic state and function of cells (Song *et al.*, 2020). Polycaprolactone (PCL) is a biocompatible and biodegradable material approved by the FDA and has been widely used in biomedicine (Aldemir Dikici *et al.*, 2020). PCL has advantages in repairing the defects of hard and slowly regenerating tissues (such as bone), but it lacks biological activity (Jirofti *et al.*, 2020). In this study, a 3D printed PCL scaffold with a pore size of 400 μm and a line width of 300 μm was used, which is conducive to bone and blood vessel formation (Hsieh *et al.*, 2005; Otsuki *et al.*, 2006). After ST2 seeded on the PCL scaffold in basal medium and 1:4 ratio of Wnt3a conditioned medium to basal medium for 3 days, the decellularized matrix modified PCL scaffold was obtained through the freeze-thaw cycle and DNase decellularization method. Then new seeded ST2 was cultured on these scaffolds *in vitro* to evaluate osteogenic performance. It was found that Wnt3a-ST2-dCM-PCL has good osteogenic and mineralization effects. This may be due to the activation of the classic Wnt signaling pathway that confers the scaffold biological activity, but the key components are still unclear. Bone regeneration is a complex process involving multiple factors. DCM ornamented scaffolds retain immunomodulatory cytokines, such as transforming growth factor-β, bone morphogenetic protein, and βFGF, which modulate pro-inflammatory responses (Zhang *et al.*, 2022). 3D-printed scaffold modified with decellularized matrix of Human umbilical cord mesenchymal stem cells (hUCMSCs) has been reported not only attenuate host rejection, but also increase the accumulation of M2 macrophages to initiate tissue regeneration and combat inflammation (Deng *et al.*, 2020). And studies have found appropriate inflammatory responses are necessary for bone regeneration and can alter tissue reconstruction after trauma (Ma *et al.*, 2017; Tatullo *et al.*, 2019). In addition, transforming growth factor beta-3 (TGF-β) and insulin-like growth factor 1 (IGF-1) have been discovered to promote meniscus-derived decellularized matrix (dCM) to induce the differentiation of human knee synovial fluid resident mesenchymal stem cells (SF-MSCs) towards a meniscus phenotype (Liang *et al.*, 2018). So future research should focus on the use of proteomics analysis to identify the composition of various cell matrix proteins and biological factors.

Studies have shown that the number of cell debris and DNA residues is the main source of adverse immune responses and may interfere with the reconstruction of biological scaffolds (Garcia and Martin, 2019; Yang *et al.*, 2020). Pati's group prepared cell decellularized matrix modified 3D printed scaffolds by freeze-thaw cycles in the hypertonic solution, which effective removed DNA as well as retained most cell matrix components and matrix structure. The scaffolds promoted cell adhesion, proliferation, osteogenesis *in vitro* and promote *in situ* and ectopic bone

formation in rats (Pati *et al*., 2015). Carvalho discovered freeze-thaw cycles combined with DNA enzymes removed more DNA than freeze-thaw cycles alone, but there was no difference in cell matrix components. The dCM can promote blood vessel and bone formation *in vivo* (Carvalho *et al*., 2019). Other studies have also proved that the freeze-thaw cycle-DNase decellularization method effectively removes DNA while retaining the cell matrix (Li *et al*., 2019b; Londono *et al*., 2017). In this study, the quantitation of DNA and total protein showed that freeze-thaw cycles and DNase removed cellular DNA while retaining most of the cellular matrix components. Scanning electron microscopy images of the PCL scaffold ornamented with decellularized matrix before and after decellularization also clearly showed that the cells on the scaffold grew well before decellularization, and a large amount of cellular matrix was attached to the scaffold after decellularization, with almost no cell morphology. This also further showed that the decellularization method used in this study ensures the accuracy of the experiment.

ST2 was seeded on the dCM ornamented PCL scaffolds and live and dead staining showed that the cells survived about 90% after 24 h. The proliferation of ST2 on the Wnt3a-ST2-dCM-PCL scaffold was significantly stronger than that of the other two groups. We speculate that the ST2 decellularized matrix induced by Wnt3a promotes the faster proliferation of ST2. At the same time, ST2 cultured on the Wnt3a-ST2-dCM-PCL scaffold for 7 d and 14 d osteogenic marker genes expression was significantly increased compared with the other two groups. After 14 days of osteogenic induction, the calcium deposition also increased significantly. These results all prove that the Wnt3a-ST2-dCM-PCL scaffold has good biocompatibility and promotes cell proliferation, early osteogenic differentiation, late mineralization.

The results of this study showed that Wnt3a-induced ST2 decellularized matrix ornamented 3D printed PCL scaffold promoted ST2 proliferation, osteogenic differentiation, and mineralization. And there are sufficient sources of ST2 and Wnt3a. Therefore, the ST2 decellularized matrix induced by Wnt3a can be mass-produced *in vitro* and is a potential bioactive bone repair material. However, there are some factors that hinder the transformation of dCM to clinical practice. Firstly, the most suitable cell type for bone regeneration therapy is still unclear. Although mesenchymal stem cells (MSCs) have great potential for cell-based tissue repair and regeneration therapy and are widely used in clinical and experiment for their proliferative, multidirectional potential, immunomodulatory and anti-inflammatory effects, the risks still exist (Bejleri and Davis, 2019). Autologous human periapical cyst enchymal stem cells (HPcy-Mscs) have been found to enhanced proliferation, cell viability and gene expression of osteogenic and odontogenic differentiation of scaffold materials (Tatullo *et al*., 2019). Therefore, autologous mesenchymal stem cells will be a good cell type of dCM for bone tissue engineering and regenerative medicine, which will be the focus of our future research. What's more, the decellularization protocol should be optimized to minimize immunogenic substances and toxicity and to maximize the preservation of cellular matrix components. The difficulty in standardizing decellularized methods also limits clinical transformation (Zhang *et al*., 2022). The control of scaffold degradation requires precise modification. If the scaffold degrades rapidly, mechanical failure may occur. On the contrary, if the scaffold degrades slowly, it may activate the inflammatory response and hinder tissue repair (Haugen *et al*., 2019). Therefore, the balance between seasonal degradation and new bone formation is imperative and has proven to be a major challenge.

In summary, despite the shortcomings mentioned above, dCM is acknowledged as an ideal choice for bone tissue engineering. Besides, Wnt3a-induced ST2 dCM could provide a biomimetic microenvironment for integrating with stem cells and other bioactive materials, which is a promising inspiration for the design of future bioscaffolds as well as an optimized solution to problems in regenerative therapies. However, further research is needed to study the dCM ornamented scaffolds' bone regeneration *in vivo* and the host's response, optimize the amount of decellularized matrix on the scaffold, and analyze the specific components of the dCM for clinical practices.

Conclusions

We successfully constructed Wnt3a induced ST2-dCM ornamented porous PCL scaffolds with a defined structure and enhanced biological performance which induced osteogenic differentiation and mineralization of stem cells. Many studies have induced specific cell functions by decellularized matrices of various cultured cells, and attempted to develop preparation methods that maintain the matrix microstructure and improve the ability of the matrix to induce specific cell functions. There are still many issues to be resolved regarding matrix preparation methods, cell sources, mechanisms and standardization of clinical use. However, cultured cell-derived decellularized matrix has many advantages in inducing specific cell functions. It is hopeful that the development of decellularized matrices derived from cultured cells will be further developed in the future. All contributors who do not meet the criteria for authorship should be listed in this section.

Acknowledgement: All contributors who do not meet the criteria for authorship should be listed in this section.

Availability of Data and Materials: All the datasets used in this study are available from the corresponding author upon reasonable request.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: Xiaolin Tu; data collection: Xiaofang Wang, Yufei Ma, Jie Chen, Yang Song, Guangliang Liu; analysis and interpretation of results: Xiaofang Wang, Yufei Ma, Jie Chen; draft manuscript preparation: Xiaofang Wang, Yufei Ma, Jie Chen, Xiaolin Tu. All authors reviewed the results and approved the final version of the manuscript.

Funding Statement: This work was supported by the National Natural Science Foundation of China (Grant No. U1601220), the National Natural Science Foundation of China (Grant No. 82002310), the Chongqing Postgraduate Research and Innovation Project (Grant No. CYB20167),

the Chongqing Postdoctoral Science Foundation (Grant No. csts2019jcyj-bsh0068).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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