Creating a Living Hyaline Cartilage Graft Free from Non-Cartilaginous Constituents: An Intermediate Role of a Biomaterial Scaffold

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A novel living hyaline cartilage graft (LhCG) with controllable dimensions and free of non-cartilaginous constituents for articular regeneration is developed. As a living graft for regenerative medicine, LhCG is purely living tissue based and truly scaffold-free. The process of neotissue formation in LhCG is mediated by an interim biomaterial-based novel scaffolding system. This design highlights a philosophy of using biomaterials in engineered regenerative medicine as a transient guiding facility rather than a permanent part of substitute. The fabrication is designed and practiced in a continuous and integrated process, which attributes to its simplicity in operation. Because of the intrinsic non-cell-adhesive property of hydrogel scaffolds, articular chondrocytes' phenotype is always preserved throughout the whole procedure, which has been tested and approved both in vitro and in vivo. In situ grafting trials in a rabbit model showcase high success rates in both cartilage repair and graft-host integration. Beyond cartilage repair, this LhCG model may provide a living-tissue-based open platform or niche for multi-tissue regenerations.

1. Introduction

Articular hyaline cartilage is a delicate tissue composed of specific cells, chondrocytes, and specific extracellular matrix (ECM), collagen type II (Col II) and proteoglycans.[1–3] Its superior biomechanical properties, which critically depend on its constituent purity, are essential for smooth sliding and withholding of compression at the bone conjunction during movement. Articular cartilage has an avascular structure that limits its self-healing capacities once damaged. Common pathologies, such as arthritis, mechanical trauma and injuries, and even daily wear and tear, can make articular cartilage highly susceptible to lesions and thinning.[4,5] Current treatment of cartilaginous disorder relies on knee replacment or corrective therapies, such as arthroplasty, that substitute impaired cartilage with either man-made alloy grafts or natural surrogate cartilage grafts, including autografts, allografts, and xenografts.[6] In addition to the shortage of graft sources, given the nature of being a hard tissue, these implanting procedures commonly suffer from poor integration of grafts with the host and result in loosening or even failure of the implanted components.[7,8] As such, clinicians and researchers have proposed to create and implant therapeutic cell-based cartilaginous constructs as programmable and controllable "living grafts" in replacement of either metallic grafting device or natural hard tissue explants.[9–12]

Encouraged by some preliminary but promising outcomes from in vivo trials with animal models, biological and preclinical scientists have popularly adopted colony forming strategies with chondrocytic cells or progenitors followed by prolonged pellet culture and expansion to pursue a piece of engineered living cartilaginous graft.[13–15] The general approach is to mimic pre-cartilage condensation during primary development, that is, to first generate chondrocyte isogenic groups (colonies) via suspended pellet culture, then condense these chondrocytic pellets into scattered nodules-like microscopic cartilaginous tissues and further assemble these pieces of microtissues into a single piece of tangible macroscaled cartilaginous tissue. This "stack-up" strategy has achieved some preliminary success but also posed a dilemma: the scattered microtissues possess a perfect hyaline cartilaginous phenotype in each piece but remain too small in size to be practically handled for any applications; on the other hand, the process of scaling up from micro- to macrotissue would inevitably cause various degrees of phenotype loss as well as introduction of impurities, typically, fibrosis due to collagen type I (Col I).

Based on this state-of-art information, rather than employing the strategy of piling up premade microtissues, we have developed a continuous methodology to directly set up a macroscaled 3D living hyaline cartilage graft (LhCG) with the aid of a biomaterial-based interim scaffolding system. Porcine articular chondrocytes are first accommodated and colonized (by forming isogenic groups) in the gel phase of microcavitary (hundreds of micrometers in diameter) alginate constructs,[16,17] then guided to outgrow the gel phase and fill up the cavities forming scattered pieces of pure microtissue "nodules" via a previously reported strategy of phase transfer cell culture (PTCC).[16,18] By further interconnecting the microtissues via their own

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proliferative expansion, an integrated 3D macronetwork of pure tissues is constructed throughout the gel bulk interpenetrating with the existing biomaterial-based scaffolds. By then, the structural integrity of the whole construct no longer relies on the alginate scaffold but upon the neotissue network so that, as an interim scaffold, the alginate hydrogel is no longer necessary and thus is completely and noninvasively removed by simple citric leaching treatment.[19] As a result, a pure cartilaginous ECM- and chondrocyte-based tangible piece of living cartilage graft, LhCG, is created (**Figure 1**a–c).

2. Results and Discussion

2.1. Establishing LhCG Prototype

Given the critical contribution to constructional integrity and tissue development in LhCG, the importance of the initial cavities in gel bulk is evaluated in the chondrocytes-encapsulating alginate samples with cavities in gel bulk (namely, PTCC samples) against simple hydrogel-cell encapsulation models (namely, non-PTCC samples, without initial cavities in gel bulk) throughout 35 days of culture. Technically, for convenience of process and description in experiments and analyses, we mark this duration of cell culture within hydrogel scaffold (in PTCC and non-PTCC samples) as starting from day –35 and ending on day –0; subsequently, the removal of alginate hydrogel scaffold, that is, the appearance of LhCG happens on day 0; later, the work after alginate removal (with LhCG or controls) is timed with day +#, where # represents a day number, starting from day +0. Chondrocytes in the PTCC samples exhibits higher cell proliferation rate throughout this time period than the cells in the non-PTCC model (Figure S1a, Supporting Information). Comparing the gene expression of cells in different samples, Col II and aggrecan expression in PTCC samples were 89% and 57% higher than those in non-PTCC samples on day –14 respectively; expression of cartilage oligomeric matrix protein (COMP) was 78% higher and Sox 9 was 36% higher. These gene expressions were also compared in parallel with chondrocytes in native cartilage (NC) and 2D monolayer cultures. Not only did we see a consistent higher expression of typical hyaline cartilage markers in PTCC sample than that in non-PTCC (Figure S1b, Supporting Information), but we also found the gene expression level was similar to that in the NC. This is in line with the tendencies observed from previous PTCC studies in agarose system.[16] In addition, Col I expression was assessed to examine the presence of fibro-cartilaginous impurities. The down regulation of Col I (in applied passage 1 chondrocytes) from day –35 to day –0, further confirmed that the alginate hydrogel based 3D culture condition, is capable of favoring a relatively pure hyaline cartilaginous phenotype with minimum fibrosis. Consistently, on a biochemical level, higher glycosaminoclycan (GAG) and total collagen contents were also found in PTCC sample (Figure S1c, Supporting Information).

After these 35 days of culture (on day 0), the formation and interconnection of all micro neotissue nodules with abundant ECM have formed a neotissue-based 3D network with the integrity, which is sufficiently strong to withhold the removal

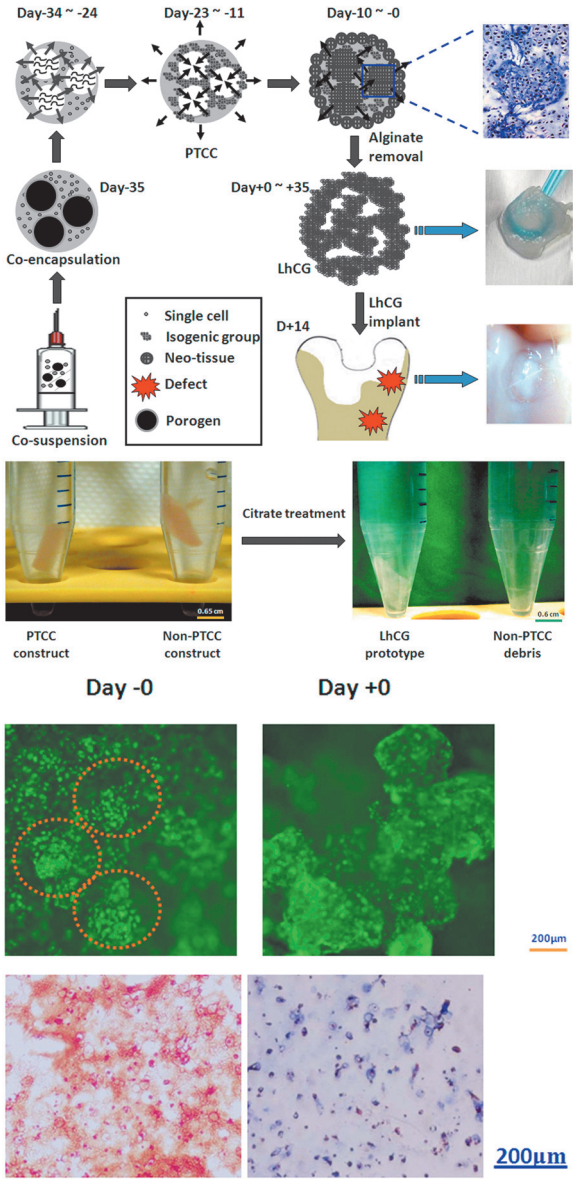

Figure 1. Preparation of LhCG. a) Schematic illustration of LhCG fabrication process. Clockwise from left bottom: Porcine chondrocytes are co-encapsulated with gelatin microspheres (porogen) in alginate hydrogel. After gelation of alginate, the dissolving of gelatin microspheres creates cavities within the gel bulk. PTCC[16,18] take place giving rise to micro tissue nodules within alginate hydrogel scaffold. The microtissues interact and secrete ECM extensively, forming an intricate interpenetrating network of ECM and hydrogel. With removal of alginate scaffold, the structural integrity of the construct remains intact and a pure, scaffold-free LhCG is formed. b) Gross overview of PTCC and non-PTCC construct before and after alginate removal via sodium citrate treatment on day 0. The structural integrity in the PTCC construct, which therefore formed LhCG construct was maintained after alginate removal, but non-PTCC collapsed into debris. c) Live/dead staining indicated the formation of neotissue nodules in PTCC sample before gel removal at day –0 and LhCG sample right after gel removal at day +0. d) Histochemical staining of PTCC sample after extended culture without alginate removal. From left to right: Safranin O, Masson's Trichrome staining. PTCC sample (day +70, totally 105 day cultivation without gel removal) showed weak and discontinuous stainings for GAG and collagen suggesting alginate removal is mandatory for further tissue development as that in LhCG.

of the hydrogel scaffold. The following removal of alginate via citrate treatment serves two critical purposes: ensuring high purity of hyaline cartilage composition free of any non-cartilaginous constituents, specifically alginate scaffolding material in this occasion in the obtained LhCG, that is, being truly "scaffold-free" and simultaneously making more living spaces for cell proliferation. The space left behind by the alginate scaffold would, again, allow further cell growth and expansion in LhCG. Conversely, in PTCC constructs, if alginate-leaching step was omitted, progressive growth and expansion of the formed 3D network of micro-neotissues would be prohibited and even degenerated due to a long-term space constraint imposed by the hydrogel scaffold (Figure 1d).

2.2. LhCG+ Working System

To further address the challenge of insufficient cell number in engineered implants for cell-based therapy, we attempted to optimize LhCG by increasing overall cell density via seeding of extra chondrocytes. Given the porous nature and the presence of rich ECM proteins in LhCG, it is a favorable living platform for additional cell seeding after removal of alginate on day 0. LhCG with cells added is named "LhCG+" while the ones without cell addition are referred to as "LhCG". To distinguish the newly added chondrocytes from existing cells in LhCG, the former were labeled with green fluorescent protein (GFP) (**Figure 2**a). Albeit the initial (up to day +3) increment in total cell number by new cell seeding was not significant, only very small percentage of which were physically held in LhCG+ pores, extremely active proliferation of this residing population (of newly added cells) boosted the cell density (in the open space of LhCG+ pores) significantly later on. (Figure 2a). Scanning electron microscopy (SEM) revealed a porous structure in both LhCG+ sample and native cartilage (NC) (Figure 2b). Compared to NC, LhCG+ samples had pores that were better connected and larger. Generally, addition of chondrocytes reinforced the existing hyaline cartilaginous phenotype in which potential fibrocartilage formation remained minimal (by gene expression and biochemical assays) and unobservable with immunostaining. (Figure 2c–e). Specifically, LhCG+ showed a higher gene expression of typical chondrocytic markers as well as higher production of proteoglycan and Col II in comparison with LhCG. Notably, when ECM production (both GAG and collagen) was normalized to the general (wet) volume of LhCG or LhCG+ construct, ECM densities per piece of these engineered tissues were approximately a quarter to an half of that in (porcine) NC, although the value from LhCG+ was still significantly higher than that from LhCG samples. Fibrosis was minimal in both LhCG and LhCG+ constructs, as seen from the very low gene expression and observably negative immune-staining of Col I. (Figure 2c–e). The generally pure maintenance of hyaline cartilaginous phenotype in adopted chondrocyte culture (CC) medium was also confirmed by significant down-regulation of osteogenic markers. (Figure 2c).

According to mechanical traits, though NC had the greatest dynamic storage (E') and loss (E'') moduli values across all frequencies, LhCG+ (day +35) sample had higher values of E' and E'' than those of all other control samples such as PTCC samples (without gel clearance) and acellular alginate hydrogels. The greater phase angle, tan δ, further indicated that both PTCC sample and LhCG+ were relatively more heterogeneous in composition and structure than NC or plain alginate gel (Figure 2f), which is due to the phase separation made by the neotissue network against the gel bulk (in PTCC) or voids (in LhCG+, leftover from alginate removal). Collectively, these data indicated a porous structure in LhCG+; it has stronger mechanical properties compared to PTCC counterpart or acellular alginate gel but remains less stiff than NC. The difference in mechanical properties between LhCG+ and PTCC sample is also due to whether further neotissue development was yielded during the extended culture with or without removal of alginate scaffold at day 0.

As a cartilaginous graft, we have maximized the cellular components in LhCG+, while competent mechanical properties and good integration with the recipient of implants are always key demands to be fulfilled.[7,8] Superior mechanical properties in NC are derived from its intricate and abundant ECM proteins;[20] however, such ECM accumulation could hardly be achieved or maintained under static in vitro culture. This is because more ECM synthesis demands high nutrient supply, but this supply is increasingly hampered due to progressively poorer nutrient diffusion as ECM accumulates. Since the clearance of alginate and along with the development of LhCG, the demand for nutrient kept increasing while the nutrient supply via medium diffusion kept decreasing. When the demand and the supply reached equilibrium, net tissue development was halted and no additional ECM deposition would take place. In LhCG+, this equilibrium point was reached earlier than day +18 as evident from the plateau starting around day+18 in the biochemical analyses of GAG and collagen content per volume (Figure 2d) and practically, also in the interest of processing time, we adopted a time window around day +14 as the equilibrium reaching period.

Given this limitation in static culture in vitro, as for possible solutions, studies have established a relationship between mechanically simulated dynamic cultural strategies with increase of ECM production and improvement of mechanical property in engineered tissues.[20] It has been proved that cyclic compression induces fluid convection and enhances transport of solutes and proteins within cartilage and consequently complements ECM production.[21–23] Similar findings were also obtained in engineered cartilage constructs under dynamic cultural conditions.[22,24] With such ECM protein accumulation, a hard and stiff engineered construct can be attained in vitro. However, it poses tremendous complexity in the simulation and exertion of proper mechanical stimuli to the engineered cartilage constructs cultured in vitro. Even if this dynamic fabrication issue can be circumvented well, implantation of an already hardened cartilage graft still faces greater problems in post-operational integration with native tissue in recipient due to the segregation by hard-to-hard interfaces,[14] which is comparable to the predicament for the natural cartilaginous explants based grafting mentioned previously.

Therefore, we proposed that instead of subjecting LhCG+ to any mechanical stimuli in an in vitro cultural environment so as to accumulate ECM and harden the graft before

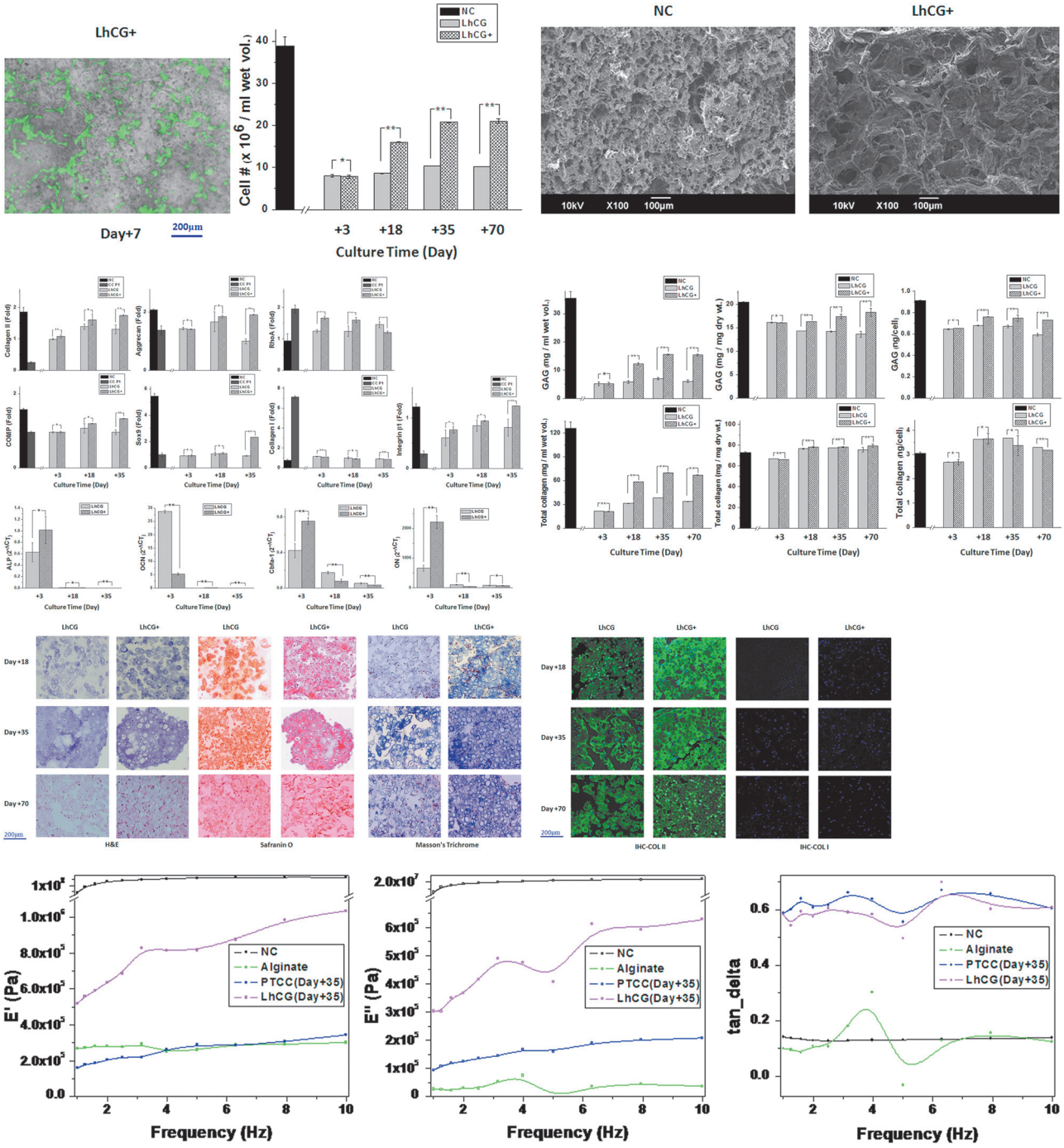

Figure 2. LhCG+ containing additional chondrocytes that were seeded onto LhCG after alginate removal on day 0 versus "empty" LhCG without cell addition. a) Left: GFP-labeled, newly added chondrocytes were used to illustrate that cell seeding was feasible in LhCG+. Right: Cell density in LhCG+ versus that in LhCG. b) SEM image of NC and LhCG+ (day +35), revealing the porous morphology of both NC and LhCG+. c) Gene expression showed higher expression of chondrocytic markers in LhCG+ and low expression of Col I indicating minimal fibrosis. All fold values were calculated based on gene expression value of PTCC samples at day –0 (indicated in Figure S1b, Supporting Information), set as unit 1. "CC P1" refers to passage 1 (P1) chondrocytes cultivated in CC medium. Osteogenic markers adopted as controls include alkaline phosphotase (ALP), osteocalcin (OCN), cbfa-1, and osteonectin (ON), down-regulation of which indicates negative tendency of osteogenesis under LhCG+ under CC condition. d) Biochemical analyses revealed GAG and total collagen production, in which LhCG+ had significantly higher GAG and collagen content per volume than those in LhCG. e) Histochemical and immunochemical (IHC) staining of LhCG+ and LhCG samples indicated a higher cell density and compact structure in LhCG+; higher GAG and Col II (positive green fluorescent signal indicates positive Col II) in ECM were also present in LhCG+ with minimal or null Col I (undetectable red fluorescence indicates minimal Col I). f) Dynamic mechanical analysis of LhCG+ (day +35) compared with NC, acellular alginate construct and PTCC sample (day +35). The obtained values represent the means ± standard deviations (sample number $N = 3$). *Differences between different constructs at the same culture time are not significant ($p > 0.05$); **Differences between different constructs at the same culture time are significant ($p < 0.05$).

implantation, LhCG+ could be directly implanted in situ into recipients on the day of equilibrium point (between days +14 and +18) without any mechanical pretreatment during the in vitro culture; the hardening process could take place naturally in the recipient body by natural biomechanical stimuli in situ after implantation. By adopting this order of preparation, LhCG+ remains relatively softer in porous sponge-like form so that it could result in better integration with surrounding tissues due to a better contacting of softer interfacing. In situ biomechanical stimuli are to be naturally exerted by the recipient during body movement after the implantation surgery. By this means, it also shortens the in vitro cultural period therefore reducing the overall fabrication time or any other processing related negativities.

2.3. In Vivo LhCG+ Subcutaneous and In Situ Implantations

Before in situ implanting trials were carried out in rabbit knee joints, LhCG+ samples were subcutaneously imbedded in nude mice for biocompatibility evaluation. We selected LhCG+ samples from day +14 and day +35 respectively. All six experimental nude mice survived the surgical procedure and the samples were harvested after 3 weeks of subcutaneous incubation in vivo. Comparing between LhCG+ samples of two different time points, both LhCG+ samples had similar ECM content consisting of abundant GAG and collagen (observably abundant Col II and few Col I; **Figure 3**a). Notably, since the samples in subcutaneous implanting positions do not receive substantial mechanostimuli, the ECM content in samples of day +14 and day +35 were not significantly different, again affirming that the equilibrium point was reached approximately day +14.

Day +14 LhCG+ samples were then used for in situ implantation to test its practical performance as a cartilage graft in rabbit knee joint model. Two superficial osteochondral defects were created on patellar groove of each femur in each rabbit's hind leg. The implants were collected 50 and 100 days after the surgery, respectively. The gross overview of the defects showed that a general healing was achieved by LhCG+ grafting (Figure 3b) and there is good developmental integration of implants with adjacent tissue once anchored well initially (in ≥80% of the defects/animals). Histological examination showed that chondrocytes in grafted LhCG+ at the first harvesting point (day 50) generally possess bigger lacunas than those in the rabbit NC. Physical integration between LhCG+ and surrounding tissue appears favorable where the ECM was well connected at a diminishing boundary between porcine cell-based LhCG+ and the adjacent rabbit cartilage tissue (Figure 3c). After 100 days of implantation, such a boundary disappeared. Host cells and tranplanted porcine cells could not be distinguished from each other in the grafting area by histological indications. Moreover, strong staining of safranin-O, masson's trichrome, and immunohistochemical (IHC) for Coll II indicated the preservation of hyaline cartilage phenotype throughout the in situ implantation period, while undetectable IHC-Col I staining proved minimum or no fibrosis at the same time (Figure 3c). Cell number analysis (normalized per wet volume) revealed that the cell density in LhCG+ grafting areas is ≈60% to 75% of that in surrounding host (rabbit) NC (Figure 3d). Biochemical quantification of GAG and total collagen had parallel outcomes with the histological analysis where high GAG and collagen content were present in LhCG+ grafting areas (Figure 3d). If normalized to the constructs' dry weight or cell number, the ECM abundance in grafting area was equivalent to that in porcine or rabbit NC. When normalized to the volume of the construct, the deduced ECM densities in LhCG+ grafting areas were comparable with that in rabbit NC even though it still remained lower than porcine NC (Figure 3d). In comparison with the ECM density (normalized by wet volume) generated in LhCG+ under in vitro static culture (Figure 2d), clearly this significant improvement was achieved due to the natural conditions in situ. Further negative controls are provided from long-term (day +70) in vitro LhCG and LhCG+ samples (Figure 2e): tissue deposition declined significantly, comparable to the long-term PTCC samples (without gel removal; Figure 1d).

Even though LhCG(+) in this study is derived from porcine (P1) chondrocytes, in situ implantation of this graft into rabbits' knee joints did not provoke any hyperacute or observable host immune rejections or reactions at the grafted area. From blood testing, only an elevation of eosinophils was detected in two out of five rabbits at day 50; while all parameters were normal in the five rabbits at day 100 timepoint (data not shown). Collectively, although these results may not exclude the potential existence of chronic host immunorejection reaction,[25] an apparent success in grafting and integration of LhCG to the host tissue was achieved. It is believed that the avascular and aneural microenvironment in articular cartilage, together with physical segregation between host cells and in vitro subcultured exotic cells by condensed cartilagious ECMs, allows accommodation of such engineered xenografts. Comparable cartilaginous xenografting experiments have been reported with similar results indicating low or minimal, or even null host immune reactions.[26–28]

3. Conclusions

In summary, all results demonstrate that the development of LhCG systems succeed in scaffold-free culture of transplantable cartilaginous tissues. This continuous methodology, which directly builds up a macro 3D living cell-based construct with the aid of an interim biomaterial scaffold, overcomes potential fibrosis during the scaling-up process from micronodules to tangible tissue. The complete and noninvasive removal of scaffolding materials ensures the high purity of LhCG and also facilitates its further development by endowing further living spaces for cells. The efficacious repair and favorable integration by LhCG upon host tissues achieved in rabbit model indicated advantages of LhCG as a promising cartilaginous graft. Furthermore, with a living tissue based nature, LhCG may also play a role as an open platform or niche for multitissue regenerations, while, for future clinical applications, experimental trials with specific pathological models in greater sized defects of larger and more active animal or human hosts with successful technotranslation toward clinical good manufacturing practice (GMP) are to be performed.

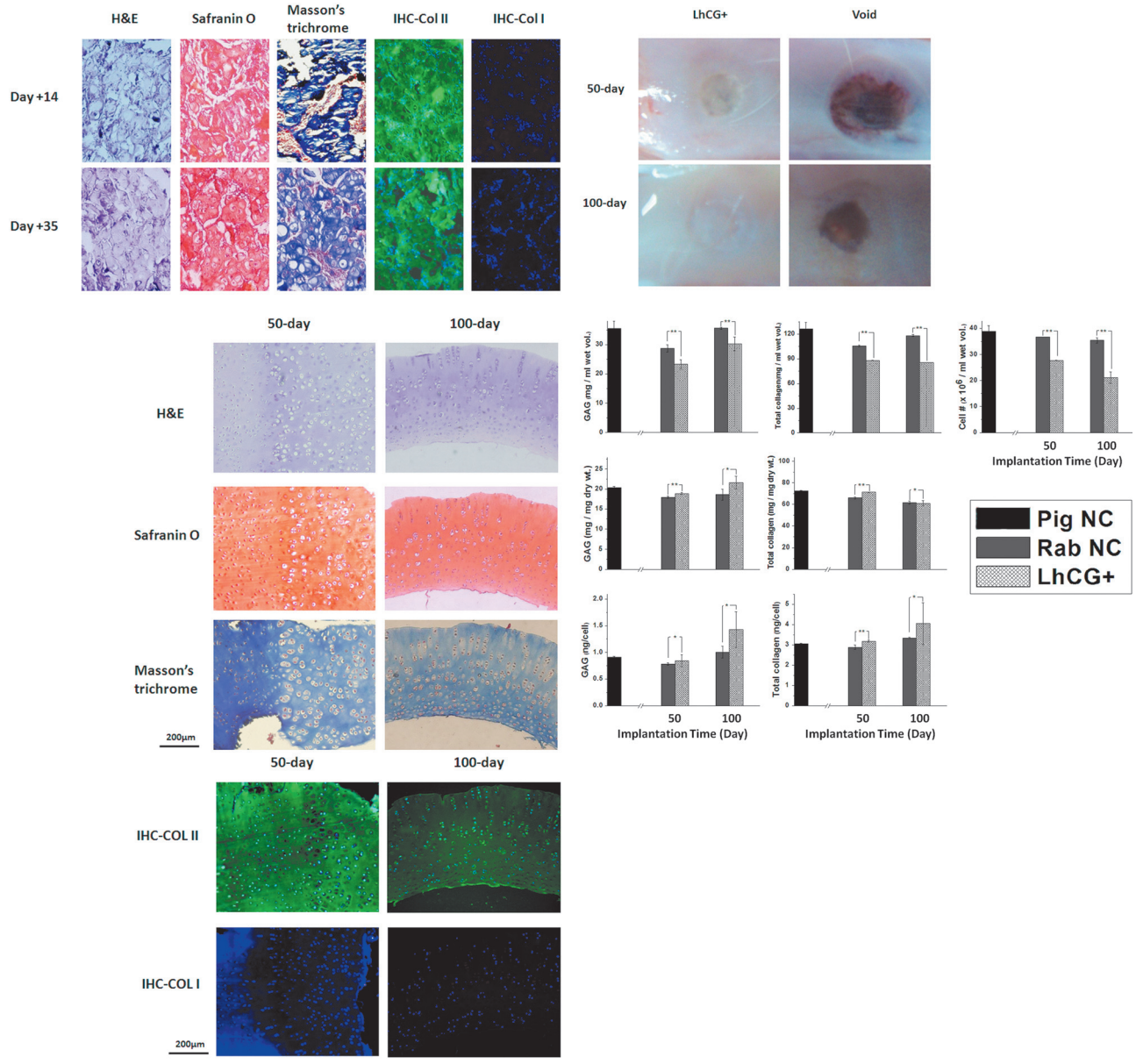

Figure 3. Evaluation of LhCG+ performance in vivo. a) Histochemical and immunochemical (IHC) staining of LhCG+ (day +14 and day +35 samples) after 3 weeks of subcutaneous implantation in nude mice (total 6) indicated maintenance of hyaline cartilaginous phenotype in LhCG+. b) In situ implantation of LhCG+ in the hind leg knee patella groove of New Zealand white rabbits: implanted and void control at day 50 (in 5 rabbits) and day 100 (in 5 rabbits) of post-implantation. c) Histochemical and IHC staining of LhCG+ at day 50 and day 100 post implantation revealed a good maintenance of hyaline cartilaginous phenotype with minimal or null fibrosis (green fluorescent signal indicates positive Col II and undetectable red fluorescence indicates minimal or null Col I) as well as good grafting integration. There is considerably distinct boundary between graft and host tissue shown after 50 days of implantation. There is absence of such boundary in LhCG+ after 100 days of implantation. d) Biochemical analyses indicating cell number, GAG and total collagen content in LhCG+ (from grafting area), and rabbit NC at day 50 and day 100 post implantation, respectively, as well as in porcine NC. The obtained values represent the means ± standard deviations ($N = 3$). *Differences between different constructs at the same culture time are not significant ($p > 0.05$); **Differences between different constructs at the same culture time are significant ($p < 0.05$).

4. Experimental Section

Construction of LhCG Prototype: Gelatin microspheres were fabricated using an oil/water/oil double emulsion method. The obtained microspheres were sieved into selected spheres size in the range of 150–180 μm and washed in a 10× concentration of penicillin/streptomyocin solution for sterilization. Primary chondrocytes were extracted from porcine hind leg knee joint and only passage 1 chondrocytes were used in the construction of LhCG. They were co-suspended at a density of 1×10^7 cells mL^{-1} in 1.5 mL cold alginate solution (1.5% w/v in 0.15 M NaCl, 4 °C) with gelatin microspheres. The mixture was injected into a 35 mm petri dish. Aqueous calcium chloride (102 mM) was gently introduced to the mixture.[17] Gelation was achieved within a few minutes during which no dissolution of gelatin microspheres was observed. In parallel, the same-sourced chondrocytes was encapsulated in 1.2% alginate hydrogel with absence of gelatin microspheres via

the same protocol as a control. The constructs co-encapsulating both gelatin microspheres and chondrocytes was named PTCC; the control without gelatin microspheres was named non-PTCC. All in vitro samples (constructs) were cut to approximately 6 mm × 5 mm × 1.5 mm in size and were cultivated on pre-laid (agarose) gel surface in CC medium that was composed of Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS, Invitrogen), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES, 0.01 M), non-essential amino acids (NEAA, 0.1 mM), proline (0.4 mM), vitamin C (0.05 mg mL^{-1}), penicillin (100 units mL^{-1}), and streptomycin (100 mg mL^{-1}). The constructs were cultured on an orbital shaker with shaking every alternative 12 h at 50 rpm in 37 °C incubator. In PTCC samples, gelatin microspheres readily dissolved under elevated temperature (37 °C) and created cavities in the construct within the first 48 h. After 35 days of culture, both constructs were treated with sodium citrate (55 mM in 0.15 M NaCl) for 10 min at room temperature to remove the alginate gel phase.[19] With the clearance of alginate scaffold, LhCG constructs (prototype) were established from the PTCC constructs, while the non-PTCC constructs collapsed into pieces of debris and were discarded. This day was marked as day 0. Any reference of a day before construction of LhCG (day 0) was marked with a negative sign (e.g., day –35) and any day thereafter was assigned a positive sign (e.g., day +35).

LhCG+ Working System: Immediately after removal of alginate hydrogel scaffold, LhCG (prototype) was transferred to 3 μm pore size cell culture insert (BD Falcon), to which 200 μL of passage 1 porcine articular chondrocytes cell-suspension (5 million mL^{-1}) was added. GFP-labelled chondrocytes were only used in the initial seeding to distinguish newly added cells from existing ones in LhCG. The LhCG constructs received this further seeding of chondrocytes were referred to as LhCG+ working system and those without further cell seeding were still referred to as LhCG control. CC medium was used for the culture of both LhCG and LhCG+ on pre-laid (agarose) gel surface until day +35.

Subcutaneous Implantation of LhCG+ in Nude Mice: Nude mice (4-week-old, mutant BALB/C, i-DNA Biotechnology Singapore) were sedated using a combination of ketamine (40 mg kg^{-1}) and diazepam (5 mg kg^{-1}) and then antisepticized with iodine and 70% ethanol. LhCG+ specimens at day +14 and day +35 were implanted subcutaneously in the pockets on both sides lateral to dorsal midline. Four small incisions were made in each mouse and a total of six mice were used. Each mouse received two LhCG+ day+14 and two LhCG+ day +35 implants. The samples were harvested after 3 weeks for histological and IHC examination. All nude mice experiments were carried out in accordance with regulations of the Institutional Animal Care and Use Committees (IACUC), Nanyang Technological University (NTU), Singapore.

Rabbit Osteochondral Defect Repair: All rabbit experiments were carried out in accordance with regulations of the IACUC, NTU, Singapore, and Xiamen University, China. Ten 5-month-old New Zealand white rabbits were used in this study. The rabbits were anaesthetized with intravenous injection of 20% urethane. Bilateral chondral defects (3 mm in diameter × 1 mm in depth[7]) were produced on patella groove of the hind legs. LhCG+ samples from day +14 of same dimensions were trimmed to fit into the defects (without further immobilizing treatment). Two defects were created in each knee of rabbit's hind legs of which one was both filled with LhCG+ samples and the other was kept untreated as negative controls. To alleviate post-operative inflammation and pain, routine drugs were administered for several days following the surgery. The rabbits were allowed to move freely in their cages and their weight-bearing activities were not restricted. Signs of any normal/abnormal behavior were then observed with careful record. The blood samples were collected before the animals were sacrificed for cartilage sample harvesting from the grafting areas after 50 or 100 days of surgery (five rabbits at each time point) for biochemical, histological, and IHC examinations.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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