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# Supporting Information

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Hyperplastic Human Macromass Cartilage for Joint Regeneration

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# Supporting Information

## Supplementary Experimental Procedures

**CM ingredients verification.** All ingredients were added to DMEM/F12 as positive control. By removing one ingredient and keeping others constant, corresponding impacts on cell proliferation were identified (Table S4, Figure S2C, D). Primary chondrocytes were seeded in 96-well plate and cultured in candidate medium. The medium was refreshed at day 3. The effect was assessed by the area of cell clusters observed under the microscope (Olympus) at day 6.

**RNA extraction, reverse transcription, and quantitative real-time PCR.** Total RNA was isolated from samples using Trizol (Takara) and transcribed into cDNA using a ReverTra Ace® qPCR RT Master Mix (TOYOBO) according to the protocols. Quantitative real-time PCR was performed on the LightCycler®480 PCR System (Roche) using TB Green® Premix Ex Taq™ (Takara). The relative expression levels of each gene were analyzed using the $2^{-\triangle\triangle Ct}$ method. The forward and reverse primers for each gene are shown in Table S5.

**Scanning electron microscopy.** According to previous reports, paraffin sections were deparaffinized and hydrated [1]. After sputter coated with gold particles, the samples were imaged with a scanning electron microscopy (SEM, Hitachi SU8010).

**Cell viability assessment of macro-cartilage.** Cell viability in macro-cartilage was assessed by Live/Dead staining and trypan blue staining.

For Live/Dead staining, macro-cartilage was incubated in 20 μM Calcein AM (Dojindo) and 20 μM Cellstain- PI (Dojindo) at 37°C for 30 min. After rinsed with

PBS, the stained macro-cartilage was visualized with a confocal microscope (Olympus, FV1000).

For trypan blue staining, macro-cartilage was digested into single cells and mixed with 0.4% trypan blue. The stained cells were quantified within 3 min through automated cell counter (Countstar).

## Supplementary Figures

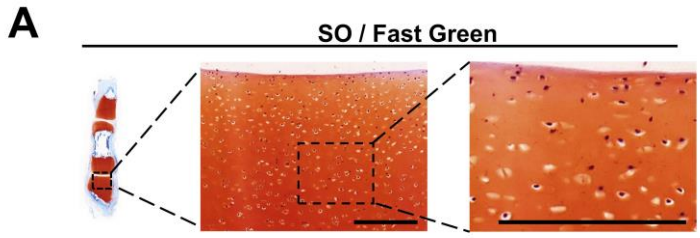


**Figure S1. The polydactyly cartilage exhibits a hyaline cartilage-like phenotype.**

(**A**) Representative images of polydactyly digital stained by Safranin- O (SO)/fast green. Scale bar: 200 μm.

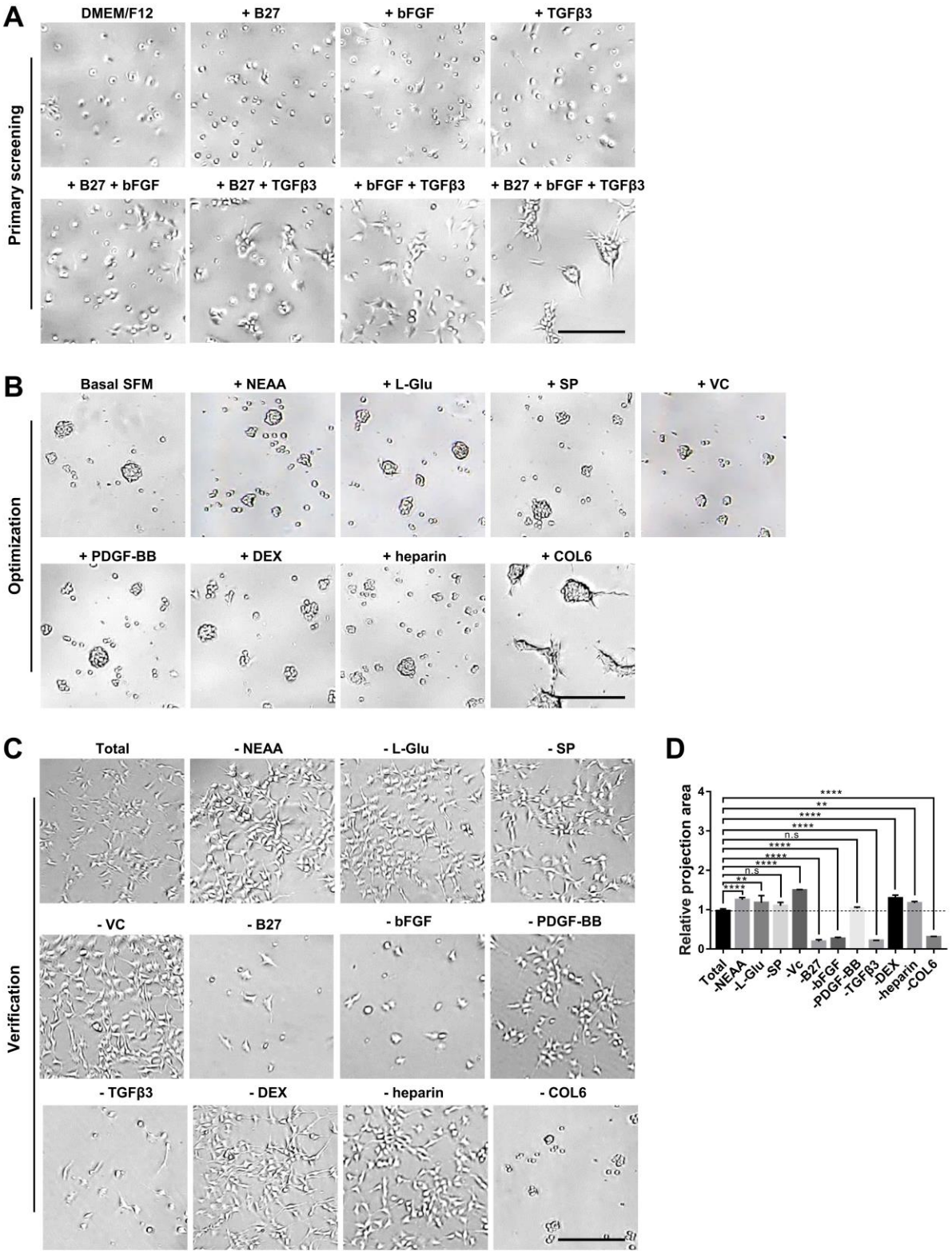


**Figure S2. A screen-defined serum-free medium efficiently expands polydactyly chondrocyte *in vitro*. Related to Figure 1.**

(**A-C**) Representative morphologies of primary polydactyly chondrocytes

cultured in different culture conditions. Scale bar: 200 μm. (**D**) Effect quantification of different additives ($n$=3, one-way ANOVA followed by Dunneett's multiple comparison test). All data were mean ± SEM. n.s $p \geq 0.05$, **$p < 0.01$, ****$p < 0.0001$.

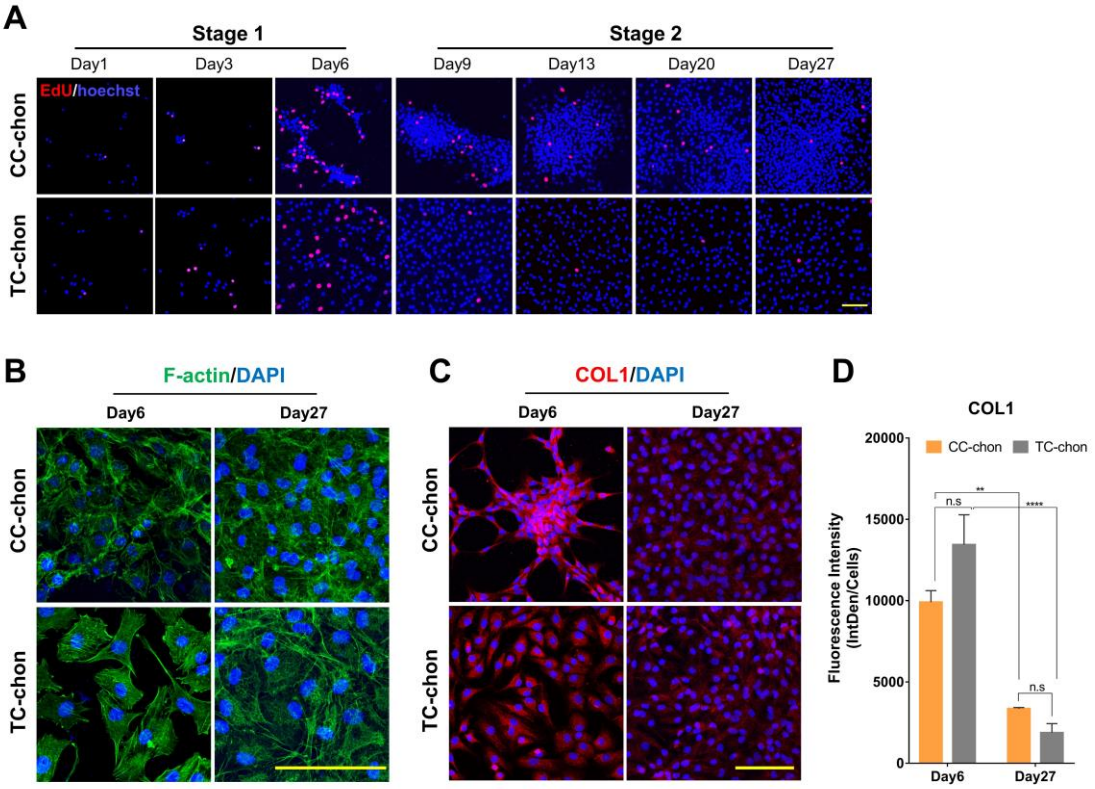


**Figure S3. Characterization of CC-chons. Related to Fig1.**

(**A**) Representative fluorescent images of proliferating cells (EdU) in CC and TC over time, DAPI labeled the nucleus. Scale bars: 100 μm. (**B**) Representative images of CC-chons and TC-chons stained with DAPI (nucleus) and Phalloidin (F-actin). Scale bars: 100 μm. (**C**) Representative images of immunofluorescent staining of COL1 in CC-chons and TC-chons. Scale bars: 100 μm. (**D**) Efficiency quantification of COL1 staining ($n$ = 6, one-way ANOVA followed by Tukey's multiple comparison test). All data were mean ± SEM. n.s $p \geq 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

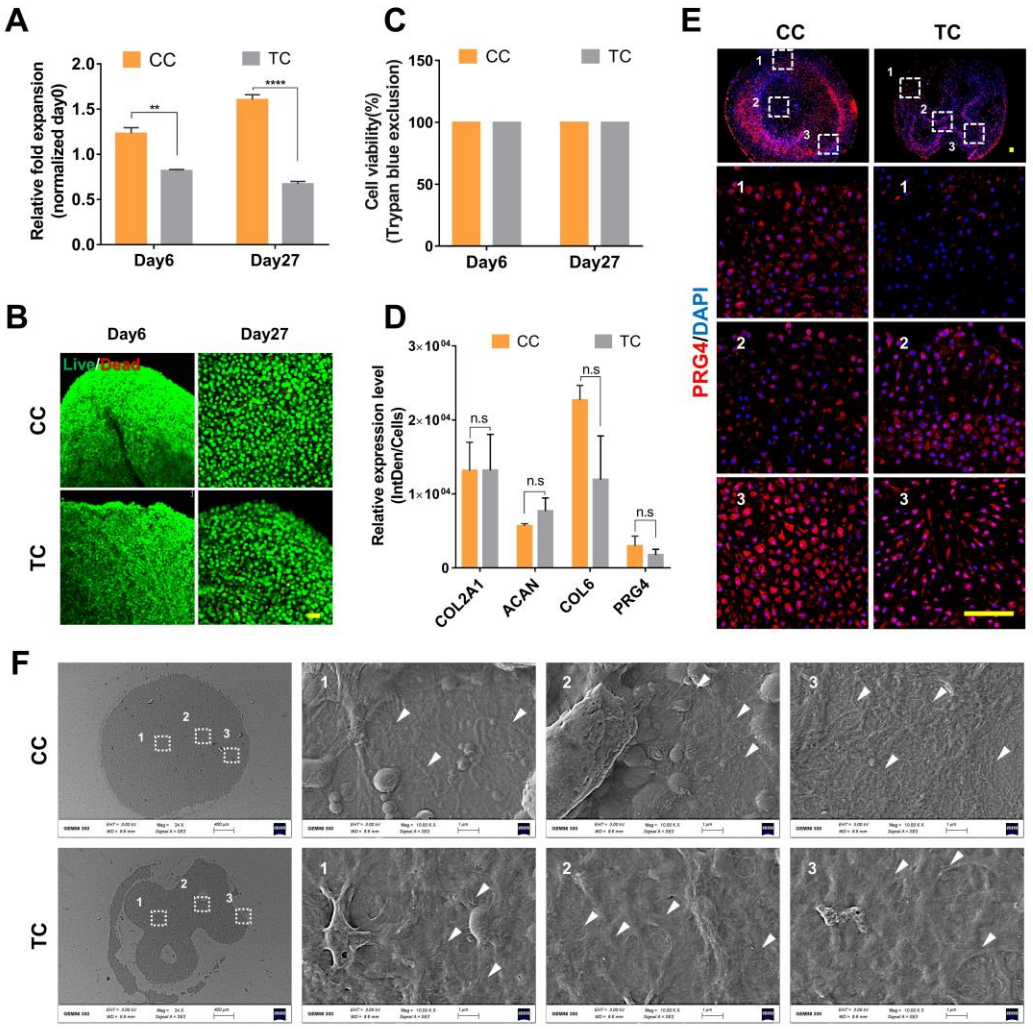


**Figure S4. Characterization of macro-cartilage. Related to Fig3.**

(**A**) Cumulative fold expansion in CC and TC culture at day 6 and day 27 (*n*=3, unpaired two-tailed Student's t tests). (**B**) Representative images of Live (green)/Dead (red) staining in CC and TC tissues at day 6 and day 27. (**C**) The percentage of viable cells in CC and TC tissues at day 6 and day 27, characterized by trypan blue staining (*n*=3). (**D**) Representative images of immunofluorescent staining of PRG4 in CC and TC tissues (*n*=3, unpaired two-tailed Student's t tests). (**E**) Efficiency quantification of COL2A1, ACAN, PRG4 staining in CC and TC tissues. Scale bars: 100 μm. (**F**) Representative SEM images of CC and TC tissues. All data were mean ± SEM. n.s $p \geq 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

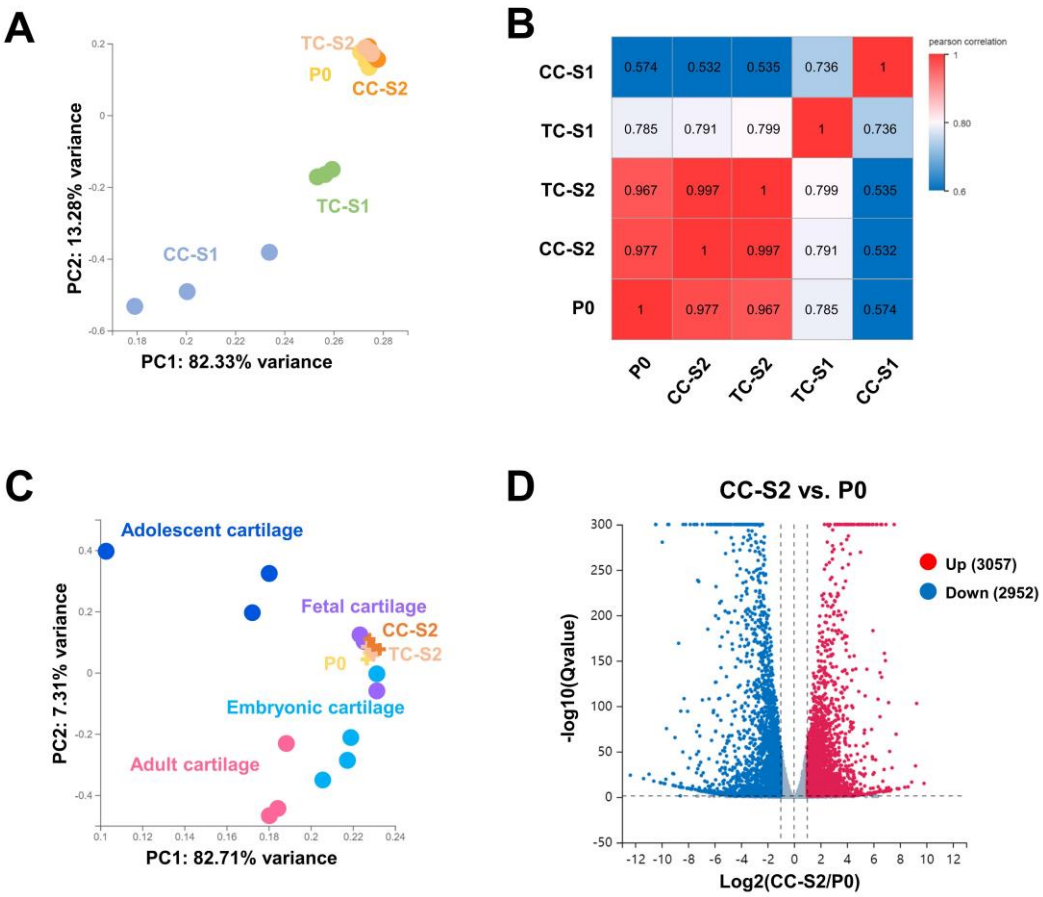


**Figure S5. Overall transcriptome analysis of macro-cartilage. Related to Fig 4.**

(**A**) Principal-component analysis (PCA) plot of all samples. (**B**) Correlation coefficient matrix of all samples. (**C**) Principal-component analysis (PCA) plot of P0, CC-S2, TC-S2 and human articular cartilage at different developmental stage. (**D**) Volcano plot identifying genes that are differentially expressed between CC-S2 and P0.

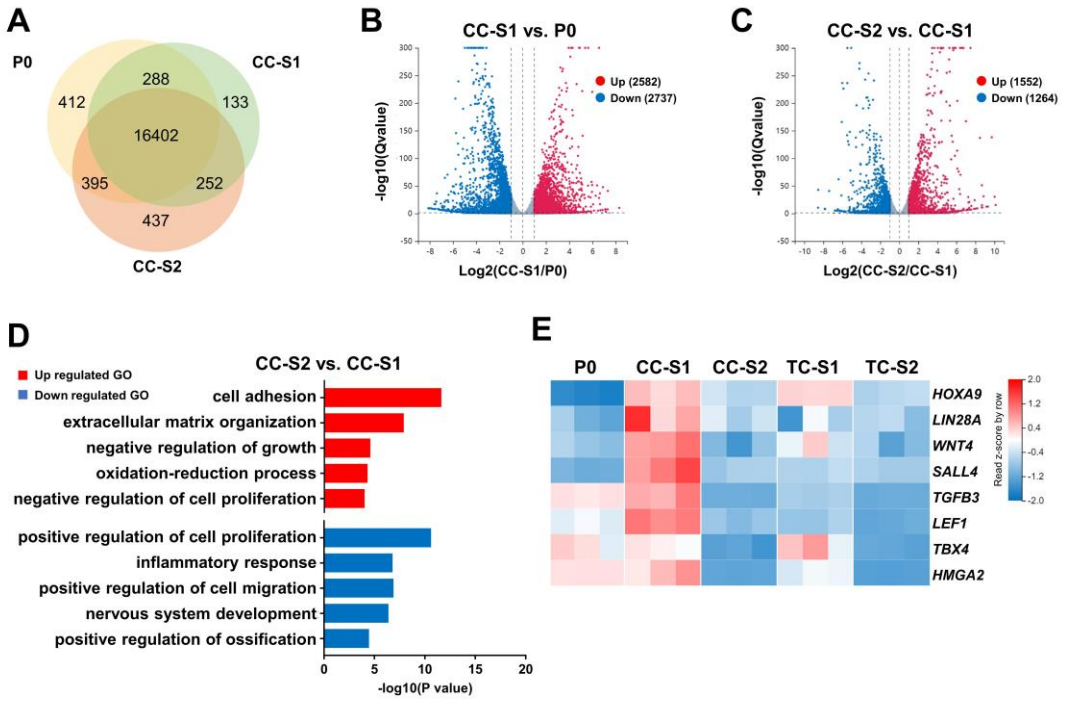


**Figure S6. CC-chondrocytes acquire an intermediate plastic phenotype in Stage 1. Related to Fig 5.**

(**A**) Venn diagram of the number of genes expressed in different stage of CC macro-cartilage (P0, CC-S1, CC-S2). (**B**) Volcano plots of differentially expressed genes between CC-S1 and P0. (**C**) Volcano plots of differentially expressed genes between CC-S2 and CC-S1. (**D**) GO enrichment analysis comparison between CC-S2 and CC-S1. (**E**) Heatmaps of genes drove early reprogramming.

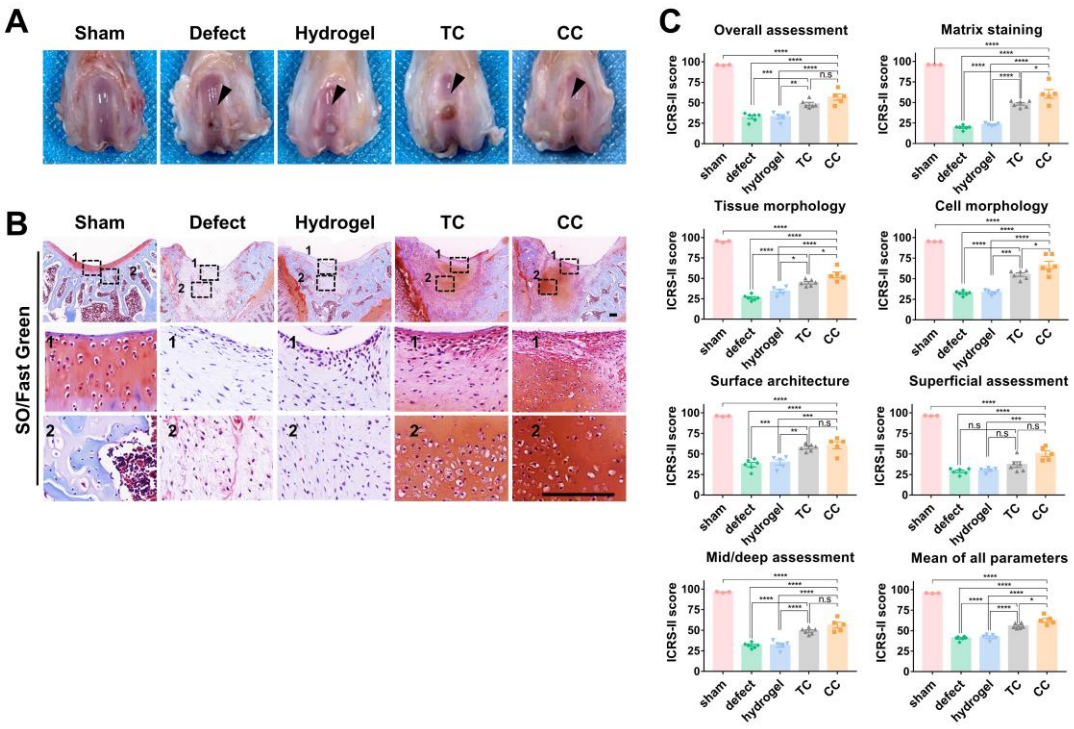


**Figure S7. Evaluation of articular cartilage repair at 6 weeks post-operatively. Related to Fig6.**

(**A**) Overall repair of cartilage defects 6 weeks after implantation. (**B**) Representative images of repaired cartilage stained by SO/fast green. Scale bar: 200 μm. (**C**) ICRS-II scoring for histological assessment of regenerated cartilage ($n$ = 3-6, one-way ANOVA followed by Tukey's multiple comparison test). All data were mean ± SEM. n.s $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

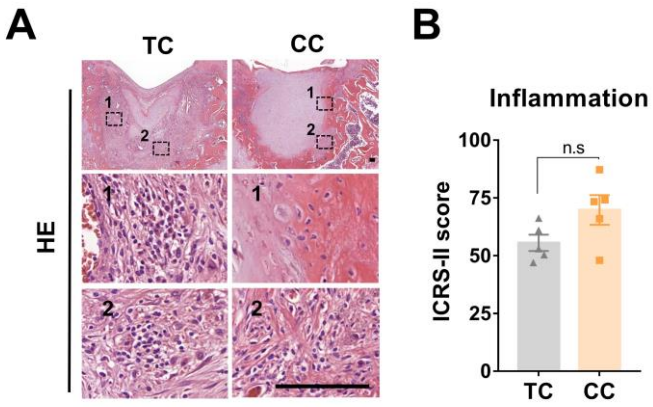


**Figure S8. CC macro-cartilage elicits a relative weaker inflammatory response. Related to Fig6.**

(**A**) Representative images of repaired cartilage stained by hematoxylin-eosin (HE). Scale bar: 100 μm. (**B**) ICRS-II scoring for histological assessment of regenerated cartilage (*n*=3, unpaired two-tailed Student's t tests). All data were mean ± SEM. n.s $p \geq 0.05$.

**Supplementary Tables**

**Table S1. Donor information of human chondrocytes.**

| Number | Age | Sex | Experiment |
|---|---|---|---|
| 1 | 11M26D | Female | Establishment of culture conditions |
| 2 | 9M24D | Female | |
| 3 | 1Y3M | Male | |
| 4 | 1Y | Male | |
| 5 | 1Y | Female | |
| 6 | 2Y | Female | |
| 7 | 1Y7M | Female | |
| 8 | 1Y1M | Male | |
| 9 | 5M3D | Male | |
| 10 | 6M24D | Male | Characterization of CC-chons in 2D culture |
| 11 | 11M | Male | |
| 12 | 7M | Female | |
| 13 | 1Y3M | Male | |
| 14 | 4M10D | Male | *In vitro* characterization of macro-cartilage |
| 15 | 1Y11M | Male | |
| 16 | 7M22D | Male | |
| 17 | 3M10D | Female | |
| 18 | 5M12D | Female | |
| 19 | 7M26D | Male | |
| 20 | 13M | Female | Bulk RNA sequencing of of macro-cartilage |
| 21 | 11M20D | Female | |
| 22 | 4M8D | Male | |
| 23 | 11M | Female | |
| 24 | 6M17D | Female | |
| 25 | 1Y1M | Female | |

| | | | |
|---|---|---|---|
| 26 | 7M8D | Female | |
| 27 | 1M9D | Male | |
| 28 | 3M5D | Female | Implantation of macro-cartilage in rat joints |
| 29 | 3M1D | Female | |
| 30 | 6M20D | Male | |
| 31 | 2M | Female | |
| 32 | 5M21D | Male | |
| 33 | 7M7D | Female | |
| 34 | 2Y10D | Female | |
| 35 | 1Y | Female | |
| 36 | 1Y | Female | |

**Table S2. Candidate supplement in combinatory screening.**

| Name | Description | Source | Catalog Number | Concentration |
|---|---|---|---|---|
| B27 | B-27™ Supplement (50×), serum free | Gibco | 17504044 | 1× |
| bFGF | Fibroblast Growth Factor-basic | PeproTech | 100-18B | 30 $ng \cdot mL^{-1}$ |
| TGFβ3 | Transforming Growth Factor-β3 | PeproTech | 100-36E | 10 $ng \cdot mL^{-1}$ |

**Table S3. Candidate additives in optimization screening.**

| Name | Description | Source | Catalog Number | Concentration |
|---|---|---|---|---|
| NEAA | MEM Non-Essential Amino Acids Solution | Gibco | 11140050 | 1 × |

| | | | | |
|---|---|---|---|---|
| | (100×) | | | |
| L-Glu | L-Glutamine (200мм) | Gibco | 25030081 | 2 мм |
| SP | Sodium Pyruvate (100мм) | Gibco | 11360070 | 1 мм |
| Vc | Ascorbic Acid | Sigma | A92902 | 50 μg·mL$^{-1}$ |
| PDGF-BB | Platelet-Derived Growth Factor-BB | PeproTech | 11-14B | 20 ng·mL$^{-1}$ |
| DEX | dexamethasone | Sigma | D4902 | $10^{-8}$ м |
| Heparin | Heparin sodium | Selleck | S1346 | 2 μg·mL$^{-1}$ |
| COL6 | Collagen 6 | Abcam | ab7538 | 2.5 μg·mL$^{-1}$ |

**Table S4. Candidate components in verification experiment.**

| **Name** | **Description** | **Source** | **Catalog Number** | **Concentration** |
|---|---|---|---|---|
| B27 | B-27™ Supplement (50×), serum free | Gibco | 17504044 | 1× |
| bFGF | Fibroblast Growth Factor-basic | PeproTech | 100-18B | 30 ng·mL$^{-1}$ |
| TGFβ3 | Transforming Growth Factor-β3 | PeproTech | 100-36E | 10 ng·mL$^{-1}$ |
| NEAA | MEM Non-Essential Amino Acids Solution (100×) | Gibco | 11140050 | 1× |
| L-Glu | L-Glutamine (200мм) | Gibco | 25030081 | 2 мм |
| SP | Sodium Pyruvate (100мм) | Gibco | 11360070 | 1 мм |

| | | | | |
|---|---|---|---|---|
| Vc | Ascorbic Acid | Sigma | A92902 | 50 μg·mL$^{-1}$ |
| PDGF-BB | Platelet-Derived Growth Factor-BB | PeproTech | 11-14B | 20 ng·mL$^{-1}$ |
| DEX | dexamethasone | Sigma | D4902 | $10^{-8}$ M |
| Heparin | Heparin sodium | Selleck | S1346 | 2 μg·mL$^{-1}$ |
| COL6 | Collagen 6 | Abcam | ab7538 | 2.5 μg·mL$^{-1}$ |

**Table S5. Primer sequences for real-time quantitative PCR analysis.**

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| *COL2A1* | GTCTGTGACACTGGGACTGT | TCTCCGAAGGGGATCTCAGG |
| *ACAN* | CTGCAGACCAGGAGGTATGTGA | GTTGGGGCGCCAGTTCTCAAAT |
| *SOX9* | GGCGGAGGAAGTCGGTGAAGAA | GCTCATGCCGGAGGAGGAGTGT |
| *FGF2* | AGAAGAGCGACCCTCACATCA | CGGTTAGCACACACTCCTTTG |
| *MMP13* | ATGCAGTCTTTCTTCGGCTTAG | ATGCCATCGTGAAGTCTGGT |
| *COL1A1* | CGATGGATTCCAGTTCGAGTAT | CATCGACAGTGACGCTGTAGG |
| *ADAMTS5* | ATCACCCAATGCCAAGG | AGCAGAGTAGGAGACAAC |
| *MATN3* | TCTCCCGGATAATCGACACTC | CAAGGGTGTGATTCGACCCA |
| *GAPDH* | TGACGCTGGGGCTGGCATTG | GGCTGGTGGTCCAGGGGTCT |

## Reference

[1] G. N. Hall, W. L. Tam, K. S. Andrikopoulos, L. Casas-Fraile, G. A. Voyiatzis, L. Geris, F. P. Luyten and I. Papantoniou, *Biomaterials* **2021**, *273*, 120820.