Supporting Information

for Adv. Mater., DOI: 10.1002/adma.202103737

Manufacturing of Human Tissues as off-the-Shelf Grafts Programmed to Induce Regeneration

Sébastien Pigeot, Thibaut Klein, Fabiana Gullotta, Steven J. Dupard, Alejandro García García, Andres García-García, Sujeethkumar Prithiviraj, Pilar Lorenzo, Miriam Filippi, Claude Jaquiery, Loraine Kouba, M. Adelaide Asnaghi, Deepak Bushan Raina, Boris Dasen, Hanna Isaksson, Patrik Önnerfjord, Magnus Tägil, Attilio Bondanza, Ivan Martin,* and Paul E. Bourgine*
Manufacturing of human tissues as off-the-shelf grafts programmed to induce regeneration
Sébastien Pigeot, Thibaut Klein, Fabiana Gullotta, Steven J. Dupard, Alejandro García García, Andres García-García, Sujeethkumar Prithiviraj, Pilar Lorenzo, Miriam Filippi, Claude Jaquery, Loraine Koub, M. Adelaide Asnaghi, Deepak Bushan Raina, Boris Dasen, Hanna Isaksson, Patrik Önnerfjord, Magnus Tägil, Attilio Bondanza, Ivan Martin*, Paul E. Bourgine*

Dr. S. P. Author 1, Dr. T. K. Author 2, Dr. F. G. Author 3, Dr. A. G-G. Author 6, Dr. M. F. Author 9, Prof. C. J. Author 10, L. K. Author 11, Dr. M. A. A. Author 12, B. D. Author 14, Prof. I. M. Author 19, Prof. P. E. B. Author 20.
Department of Biomedicine, University Hospital Basel, University of Basel, 4031, Basel, Switzerland.
E-mail: ivan.martin@usb.ch
E-mail: paul.bourgine@med.lu.se

Dr. S. P. Author 1, Prof. I. M. Author 19.
Department of Biomedical Engineering, University Hospital Basel, University of Basel, 4031, Basel, Switzerland.

S. J. D. Author 4, Dr. A. G. G. Author 5, S. P. Author 7, Prof. P. E. B. Author 20.
Laboratory for Cell, Tissue, and Organ engineering, Department of Clinical Sciences, Lund University, 221 84, Lund, Sweden.

S. J. D. Author 4, Dr. A. G. G. Author 5, S. P. Author 7, P. L. Author 8, Prof. P. E. B. Author 20.
Wallenberg Center for Molecular Medicine, Lund University, 221 84, Lund, Sweden.

S. J. D. Author 4, Dr. A. G. G. Author 5, S. P. Author 7, Prof. P. E. B. Author 20.
Stem Cell Center, Lund University, 221 84, Lund, Sweden.

D. B. R. Author 13, Prof. H. I. Author 15, Dr. M. T. Author 17.
Department of Clinical Sciences, Orthopedics, Lund University, 221 84, Lund, Sweden.

Dr. M. T. Author 17.
Department of Biomedical Engineering, Lund University, 221 84, Lund, Sweden.

Dr. P. Ö. Author 16.
Rheumatology and Molecular Skeletal Biology, Department of Clinical Sciences, Lund University, 221 84, Lund, Sweden.

Dr. A.T. Author 18.
Innovative Immunotherapies Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Hospital Scientific Institute, Vita-Salute San Raffaele University, 20132 Milan, Italy.

Keywords: Extracellular matrices, Endochondral ossification, Regenerative medicine, Bone graft, BMP2.
Extended Data Figure 1. Primary and immortalized cell sources do not offer a reproducible in vitro cartilage generation capable of undergoing endochondral ossification in vivo. **a,** Primary human bone marrow derived mesenchymal stromal cells (hBM-MSCs) and human nasal chondrocytes (NCs) can form cartilage in vitro but a substantial inter-donor variability is observed (Safranin-O staining). Scale bar = 100µm. **b,** The telomerase-based immortalization of pre-selected chondrogenic batches of primary cells (hBM-MSCs and hNCs) was associated with a loss of their cartilage forming capacity. (n=3 donors). Scale bar = 100µm. **c,** The mouse ATDC5 cell line is capable of limited but reproducible chondrogenesis (Safranin-O), also displaying hypertrophic features (Alizarin-red) after 5 weeks of in vitro pellet culture. However, following 8 weeks post-subcutaneous implantation in mice, the tissues did not exhibit any remodeling into bone. n≥3. Scale bar = 200µm.
Extended Data Figure 2. Identification of a MSOD-BMP2 clone exhibiting chondrogenic potential. MSOD cells were transduced with the lentivirus encoding the BMP-2 transgene. From the resulting MSOD-B heterogeneous population, 48 clones were isolated from which 10 could be successfully expanded and assessed for their chondrogenic capacity. Only the clone number 10 displayed evidence of cartilage matrix deposition, after 3 weeks of in vitro macromass pellet cultures. Scale bar = 100µm.
Extended Data Figure 3. In vitro assessment of MSOD (M) and MSOD-BMP2 (MB) proliferation and BMP-2 expression. a, Growth curves of M and MB cells indicate an identical proliferation rate in 2D culture (n = 3). b, The implementation of the BMP-2 transgene in MB resulted in a stable overexpression confirmed at the RNA (left, quantitative reverse transcriptase polymerase chain reaction) and protein level (right, ELISA assay of culture supernatant). (n=3). Graphs represent Mean±SD, *p ≤ 0.05, **** p ≤ 0.0001 determined by two-tailed unpaired t-tests.
Extended Data Figure 4. **Bone forming capacity of living engineered tissues.** Hypertrophic cartilage tissues were in vitro engineered using chondrogenic batches of hBM-MSCs, M and MB cells respectively. Following subcutaneous implantation in nude mice for 6 weeks, only the hBMSCs and MB tissues were capable of forming bone, as assessed by µCT imaging and Safranin-O (Saf-O) staining. (n=3). Scale bars = 100μm.
Extended Data Figure 5. Exposure to LDN193189 affects the chondrogenic capacity of MSOD-B cells without impacting their BMP-2 secretion. **a**, The LDN193189 (LDN) compound (BMP type I receptor inhibitor) was delivered during the 3 weeks culture time of MSOD-B (MB) chondrogenic differentiation. No effect on the cells BMP-2 secretion (left, ELISA from culture supernatants (SN)) or on BMP-2 accumulation in engineered tissues (right, ELISA) could be detected (n = 3). **b**, LDN exposure led to a marked reduction of the MB chondrogenic capacity, as assessed through GAG quantification (left) and Bern scoring (right) of in vitro engineered tissues (n = 3). Graphs represent Mean±SD. Statistics: Two-tailed unpaired t-test. *p ≤ 0.05, ***p ≤ 0.001.
Extended Data Figure 6. Ossicle maturity score description. In vivo retrieved tissues were graded based on the extent of bone (B) and bone marrow (BM) features identified by Safranin-O / Hematoxilin staining. Percentage areas of B and BM were manually determined over the total surface area of the tissue section using FIJI image analysis software. For each sample, three sections were assessed at various depths. A score from 0 (no B or BM) to 4 (over 90% of B+BM area) was given to each section and the average of the scores was retained as the ossicle maturity score for the whole sample. A minimum of three samples were analyzed for each experimental group.
Extended Data Figure 7. BMP-2 quantification of MSOD-BMP2 cartilage tissues upon temporally-controlled TGFβ3 exposure. The BMP-2 content in MB tissues was significantly lower when cells were not supplied with TGFβ3 during in vitro culture. Conversely, the BMP-2 content was substantially higher in tissues exposed to TGFβ3 for 1 to 3 weeks. This was correlated with an increase cartilage tissue maturity (see Fig. 3a,b). Quantification of was performed by ELISA, n ≥ 4. Graphs represent Mean+SD Statistics: ANOVA. *p ≤ 0.05.
Extended Data Figure 8. MSOD-B structural and mechanical properties. Scanning electron microscopy (SEM) image and corresponding pores’ diameter analysis carried out on 101 pores using ImageJ.
Extended Data Figure 9. Proteomic analysis of MSOD-BMP2 engineered tissues. a, Tukey box plot displaying the distribution of abundance of the 2456 identified protein, in each replicates (n≥4) of all biological groups. The box represents the interquartile range (IQR) of the 50% of protein abundance surrounding the mean protein abundance (between Q1 and Q3). The whiskers represent the value extending 1.5 IQR over Q3 or below Q1. b, Volcano plots displaying the respective q-value of each protein (y axis) obtained by comparing the protein abundance between two respective biological groups. The fold difference in content is reported on the x axis. Proteins in red indicate a significant abundance differences (q-values≤0.05). c, List of proteins previously identified as main native cartilage constituents and exploited for the similarity score analysis (Folkesson et al., 2018).
Extended Data Figure 10. In vitro and in vivo evaluation of lyophilized MSOD-BMP2 cartilage after 3 months of storage. a, Lyophilized MB tissues stored at 4°C for 3 months were analyzed histologically by Safranin-O (Saf-O) staining. b, Following the 3 months storage, tissues were implanted subcutaneously for 6 weeks in nude mice. Explants indicated efficient remodelling into bone and bone marrow, with ossicle maturity scores not differing from those of controls.
Extended Data Figure 11. The T-CUP perfusion bioreactor for 3D engineering of tissue grafts. 

a, In the T-CUP perfusion bioreactor system, the culture vessel (opaque structure) is clamped to the drive unit (black support), which induces relative movement with an internal plunger through compression and expansion of a bellow. 

b, A scaffold is fixed within the internal plunger, so that its movement induces alternating fluid flow through the developing tissue. The movement speed of the plunger is set through a control unit (not shown), placed outside of the cell culture incubator. Cells are injected in the system through a top valve, communicating with the inner space in the vessel. Medium is exchanged through a bottom valve, communicating with the outer space of the vessel.
Extended Data Figure 12. Tissue engineering paradigm shift. a, The classical tissue engineering paradigm involved patient-derived cells seeded and cultured into porous scaffolds to generate living grafts in an autologous setting. The concept typically follows a «tissue replacement» strategy, whereby the engineered graft should best match the patient defective tissue. 
b, The newly proposed paradigm -exemplified by our study- described the implementation of custom cell lines programmed for the engineering of tissues. Following devitalization, the remaining extracellular matrix and embedded factors can be stored as an off-the-shelf material, and ultimately implanted in patients without immuno-matching requirements. Here, the strategy relies on a «regeneration priming» concept, with the implanted graft providing the cues required to instruct endogenous repair. Cell lines offer personalized medicine approach through their selective genetic manipulation towards the generation of tailored and patient-specific implants. Standardization and scaling-up is further by bioreactor-based culture, paving the road to a clinical manufacturing process.