

## HUVEC-Derived Exosomes Containing miR-503-5p for Osteoporosis Therapy

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**Introduction:** Osteoporosis (OP) is a systemic bone disease characterized by decreased bone mass, damaged bone microstructure, increased bone fragility, and an increased risk of fractures. It is more common in postmenopausal women and elderly people. With China's aging population, the incidence of OP is increasing, imposing a significant burden on society and families. The development of novel drugs with high bone-targeting ability is urgently needed.

**Materials and methods:** We engineered extracellular vesicles derived from human umbilical vein endothelial cells (HUVEC-ExomiR-503-high) to contain a large amount of miR-503-5p and characterized them. We traced the ability of these extracellular vesicles to bind and target bone tissue and osteoblasts/osteoclasts using in vitro and in vivo fluorescence labeling. We also explored their functions in vivo and in vitro through bioinformatics analysis and a series of experiments.

**Results:** The HUVEC-ExomiR-503-high we constructed had the same characteristics as ordinary extracellular vesicles and could bind to osteoblasts/osteoclasts in vitro. Moreover, they exhibited better bone-targeting ability than extracellular vesicles derived from other sources of bone marrow stromal cells in vivo. Bioinformatics analysis showed that miR-503-5p is strongly associated with bone-related pathways. In vitro experiments showed that HUVEC-ExomiR-503-high effectively inhibited osteoclast-related pathways and promoted osteoblast proliferation and differentiation. In vivo experiments showed that injecting HUVEC-ExomiR-503-high into OP mice significantly increased bone density.

**Conclusion:** HUVEC-ExomiR-503-high can be used for targeted bone therapy of postmenopausal OP, providing a new approach for clinical prevention and treatment of OP.

## Conditional Knockout of Smad7 in Osteoclast Precursors Enhances Osteoclast Differentiation and Leads to Bone Loss

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Category: Tissue Engineering and Regeneration.

**Objective:** Osteoporosis is a systemic bone disease featuring with low bone density and fragility. Osteoclasts are responsible for bone resorption and are related to the onset and treatment of osteoporosis. Although the role of TGF- $\beta$ /Smad signaling pathway in bone formation has been well reported, their role in osteoclast differentiation and the effects on bone resorption remain unknown. This study is to investigate the role of SMAD7, an inhibitory molecule in TGF- $\beta$ /Smad signaling pathway, in osteoclastic differentiation and bone resorption in animal.

**Methods:** Smad7 was knock-downed by siRNA or overexpressed by lentivirus in osteoclast precursors-RAW264.7. The expression of Smad7 and its downstream molecules was determined by qRT-PCR and western blotting. The effect of Smad7 knockdown or overexpression, or TGF- $\beta$  supplement was measured by tartrate-resistant acid phosphatase (TRAP) staining. A mouse model with osteoclast-specific knockout (KO) of Smad7 was generated by crossbreeding of Smad7-flox and cathepsin K (Ctsk)-cre mice using CRISPR-Cas9. The microstructure of trabecular and cortical bone were determined by micro-CT analysis. The mechanical properties of femurs were measured by three-point bending tests. Bone formation and bone cells were observed by hard tissue histomorphometry.

**Results:** At cellular level, osteoclast differentiation was significantly enhanced after Smad7 knockdown, showing significantly higher number of osteoclasts in TRAP staining. The expression of p-SMAD3, TAK1, p-TAK1 and the ratio of TAK1/p-TAK1 was significantly increased after Smad7 knockdown, indicating that TGF- $\beta$  signaling was activated when SMAD7 expression was inhibited. An additive effect of TGF- $\beta$  on osteoclast differentiation in normal RAW264.7 was confirmed by TRAP staining. In contrast, when Smad7 was overexpressed, osteoclast differentiation was significantly inhibited, showing lower number and area of osteoclast after RANKL induction. And the expression of

p-SMAD3 and TAK1/p-TAK1 was significantly decreased after Smad7 overexpression, indicating an inhibitory effect on TGF- $\beta$ /Smad signaling. At animal level, significantly lower bone mass in the metaphysis of distal femur could be found in Smad7 KO mice compared with littermates at 8, 16, or 24 weeks. Mechanical properties including maximum load, elastic load, and stiffness were significantly reduced in the femurs of KO mice compared with those of littermates at 8, 16, and 24 weeks. Results from bone histomorphometry revealed a lower bone formation rate but higher number of osteoclasts in the metaphysis in the KO mice.

**Discussion and Conclusion:** We demonstrated that a downregulation of Smad7 promoted osteoclast differentiation, leading to bone loss and mechanical deterioration in animals. And an upregulation of Smad7 inhibited osteoclast differentiation. Current work suggests that Smad7 is strongly associated with osteoclast differentiation and bone mass maintenance, and that specific regulation of SMAD7 levels in osteoclasts may be a promising strategy for the treatment of osteoporosis.

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## Multipotent Mesenchymal Stromal Cells Display Functional Heterogeneity in Their Sensitivity to cAMP-Dependent Hormones

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**Introduction.** Multipotent mesenchymal stromal cells (MSCs) play a pivotal role in maintenance of tissue integrity and regeneration, especially in skeletal, connective and epithelial tissues. Despite similar morphology and immunophenotype these cells demonstrate remarkable functional heterogeneity in terms of differentiation abilities and clonogenic capacities. MSCs are regulated by a number of endocrine and paracrine signals, many of which activate G-protein coupled receptors (GPCRs), such as acetylcholine, adenosine, angiotensin II, angiotensin 1-7, ATP, dopamine, GABA, glutamate, histamine, noradrenaline, serotonin, and sphingosine-1-phosphate. Previously, using single-cell signaling analysis in living cells we demonstrated that Ca<sup>2+</sup>-dependent functional activity of MSC is regulated by cAMP/protein kinase A (PKA)-dependent signaling activated by  $\beta$ -adrenoceptors [Tyurin-Kuzmin et al., 2016].

In this study we explored the activation of Gs/cAMP/PKA signaling in response to adenosine, dopamine, histamine, noradrenaline, and serotonin in living human MSCs at the single cell level using PKA-Spark biosensor.

**Methods:** For single-cell registration of PKA activation in living cells in real time we used a genetically-encoded biosensor of PKA activity 'PKA-Spark'. It consists of polypeptide forming homo-oligomeric coiled-coil, consensus PKA substrate sequence or specific phosphopeptide-binding domain, and GFP as a readout. PKA-Spark evenly distributes through the cytoplasm and precipitates after PKA-dependent phosphorylation of PKA substrate motif. As a result, it forms intensively fluorescent droplets or aggregates. These droplets are accessible to protein phosphatases; thus, their forming is reversible.

**Results:** In this study we explored MSC response to hormones, which activate Gs/cAMP/PKA-dependent signaling, at the single cell level using genetically encoded biosensor PKA-Spark. For the first time we have demonstrated that about half of cultured MSCs are not able to activate the cAMP/PKA pathway, possibly due to the limited availability of adenylyl cyclases. Using this approach we showed that MSC subpopulations responding to various hormones largely overlapped, and the share of responding cells did not exceed 40%. Using clonal analysis we showed that signaling heterogeneity of MSC could be formed de novo within 2 weeks.

**Discussion and Conclusion:** Our findings on functional heterogeneity of type and format of cAMP-dependent response of particular cells may be associated with the functional activity of tissue. Phenomenon of self-organization of functional heterogeneity of MSCs may determine their ability to form cell niches in tissues, where cells can form a complex system composed of cells primarily attributing to the reception of stimuli and cells dedicated to driving tissue cell renewal.

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## Filamented Light (FLight) Biofabrication of Centimeter-scale Muscle Tissue Constructs Using Pax7-nGFP Primary Myoblasts

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**Introduction:** Pax7-positive satellite cells are promising for muscle tissue engineering due to their self-renewal and differentiation abilities, which form functional muscle fibers [1]. However, current biofabrication, such as electrospinning and extrusion-base bioprinting, face challenges in production rate and biocompatibility, and often fail to provide three-dimensional (3D) cell guidance cues. Here, we present the next generation of Filamented Light (FLight) biofabrication technology[2], which can create centimeter-scale muscle tissue constructs (lengths up to 3cm) using photocrosslinkable biomaterials. The microstructures within the FLight matrix support cell proliferation, differentiation, and formation of contractile muscle fibers.

**Materials and Methods:** Gelatin-norbornene (Gel-NB) and Thiol functionalized gelatin-thiol (Gel-SH) were mixed with photoinitiators and Pax7-nGFP primary myoblasts ( $3 \times 10^6$  cells/mL), and cell-laden constructs were biofabricated using a FLight printer (FLight3DTM Ver. 1.0). The printer uses optical modulation instability to produce microfilaments within the constructs (Figure 1a). The cross-section of the constructs was tailored via a digital micromirror device, while the length of construct was controlled by an auto-feeding system. The constructs were cultured into DMEM medium + 2% horse serum for differentiation before immunofluorescent imaging and electrical muscle stimulation.

**Results:** The Pax7-nGFP primary myoblasts demonstrated high viability (> 96%). After 1 day of culture, the cells migrated into the microchannels and proliferated. The primary myoblasts become highly aligned and differentiated after 3 days of culture. The immunofluorescent images showed the maturation of aligned multi-nuclear myotubes and the presence of Pax7-positive cells (Figure 1b, c) after 2 weeks of differentiation. The synchronized contraction of myotubes with different frequencies in FLight constructs under electrical stimulation (0.5, 1.0, and 5 Hz frequency, 3V) was confirmed.

**Discussion and Conclusion:** We demonstrated a strategy to biofabricate physiological-scale muscle tissue constructs. The cues in the FLight matrix showed efficient cell guidance properties, enabling the formation of aligned contractile myotubes. The maintenance of Pax7-positive cells after maturation highlighted its possibility for long-term regeneration and biofabrication of muscle tissues, which is promising for clinical treatment of VML.

References:[1] Christoph et al., Development 2011, 138, 3639. [2] Hao et al., Adv Mat. 2022, 34, 2204301.

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## Injectable remote magnetic nanofiber/hydrogel multiscale scaffold for functional anisotropic skeletal muscle regeneration

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**Introduction:** Injectable scaffolds as the *in vivo* strategy provide a non-invasive way to fill the irregular defect of volumetric muscle loss (VML) via tissue engineering (TE) strategy. However, most of the injectable hydrogels in previous studies were lack of the ability to control 3D cellular orientation and guide aligned muscle regeneration *in situ*, while which is necessary for anisotropic muscle regeneration and mechanical function recovery. Thus, developing scaffolds that can integrate these intricate macro- and microstructures for the biomimicking of living constructs containing complex anisotropic *in vitro* is a going challenge.

**Subjects and Methods:** Firstly, we use an advanced coaxial electrospinning-cyrocutting approach to prepare the monodisperse magnetic controlled short nanofibers (MSNFs) with a high yield. Then, we developed an injectable anisotropic MSNF/Gel nanofiber/hydrogel scaffold which consists of remote magnetic MSNFs and photocurable GelMA hydrogel for guiding cellular 3D alignment and biomimicking of living constructs with macro- and microstructures *in vitro*. Finally, we further investigated its potential for 3D aligned myofiber formation and enhancing skeletal muscle regeneration *in vivo*.

**Results:** The incorporation of MSNFs and the alignment of MSNFs under magnetic field coordinatively promoted C2C12 cells elongation and 3D cellular alignment within the different layer positions of the aligned MSNF/Gel scaffold. Moreover, the cell-laden scaffolds mimicking the anatomical structures of various skeletal muscle tissue including long muscle, orbicular muscle, and bipennate muscle were developed through the multi-step magnetic-control approaches, which demonstrated that these scaffolds were able to highly mimic the different anatomical structures of various native skeletal muscle tissues and other living constructs containing complex structures *in vitro*. The *in vivo* animal VML models study showed aligned MSNF/Gel injectable scaffold efficiently promoted the myofibers number rather than myofiber diameter in the acute period, and further regenerated aligned myofibers with a large diameter in the chronic period. The contractile force results indicated that injectable aligned MSNF/Gel scaffold enabled largely repair the function of muscle tissues.

**Discussion and Conclusion:** In summary, this approach offers a new promising tissue engineering strategy not only for the aligned myofiber formation for enhancing skeletal muscle regeneration *in vivo* but also for other biofabrication of living constructs containing complex anisotropy *in vitro*.

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## Injectable Photocurable Janus Hydrogel for Postoperative Anti-adhesions after Minimally Invasive Surgery

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**Introduction:** Anti-adhesive hydrogels have been developed to prevent the formation of postoperative adhesions. However, the successful design and preparation of an injectable hydrogel with superior tissue retention properties that can prevent adhesion formation following minimally invasive surgical procedures will be of immense clinical significance, while remaining an ongoing challenge.

**Subjects and Methods:** The photocurable catechol-grafted hyaluronic acid (HAD) precursor was synthesized, and the HAD hydrogel was formed after photocrosslinking. The in vitro asymmetric adhesion properties of injectable HAD Hydrogel were analyzed by using a lap-shear test. The assessment of preventing abdominal adhesions of HAD hydrogel was evaluated in rat models, and the application of HAD hydrogel for preventing adhesions in minimally invasive surgeries was further investigated in rabbit models. The mechanism of HAD hydrogel enabling the prevention of postoperative adhesion formation was also proved.

**Results:** This injectable HAD hydrogel showed superior tissue retention properties and favorably inhibited postoperative adhesion formation. This is the first time an injectable hydrogel has been designed via photocrosslinking to control asymmetric-adhesive capability. Our results showed that laparoscopically delivered HAD precursor acted as a wet adhesive on the injured cecum, while its outward-facing side was nonadherent after photocrosslinking. Intriguingly, the HAD acted as a physical barrier and polyanion trap to neutralize scavenger receptors, thereby inhibiting collagen deposition and uncontrolled recruitment of GATA6<sup>+</sup> cavity macrophages. Furthermore, the HAD significantly downregulated the expression of fibrosis-related and pro-inflammatory cytokines and promoted macrophage polarization.

**Discussion and Conclusion:** These results demonstrate that the controllable asymmetric adhesiveness and laparoscopic feasibility of the HAD hydrogel make it a promising injectable barrier for preventing the formation of adhesions after minimally invasive surgeries.



## Bone transport over an osteoinductive intramedullary implant fabricated by a hybrid tissue engineering construct (HyTEC) technique: evidence from small to large animal

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Background: Bone transport distraction osteogenesis (DO) is a successful surgery-driven tissue regeneration approaches for the treatment of large bone defects. However, challenging complications include prolonged consolidation, docking site nonunion, and pin tract infection remain challenging complications. To address these clinical challenges, we developed a novel osteoinductive biodegradable intramedullary (IM) nail by eluting bone morphogenetic protein-2 (BMP-2) from a biodegradable implant using a hybrid tissue engineering construct (HyTEC) technique as an adjunctive therapy.

Methods: A porous BMP-2-laden hydrogel layer was formed on the surface of the polycaprolactone-tricalcium phosphate (PCL-TCP) filaments using surface-initiated physical crosslinking of alginate followed by covalent crosslinking of gelatin methacrylate (GelMA) and polyethylene glycol dimethacrylate (PEGDMA), which was then freeze-dried. The properties of interface between filament and hydrogel were determined by scanning electron microscopy (SEM) imaging, adhesion strength testing, and contact angle goniometry testing. The release profile of the BMP-2 in the IM nail was measured after lyophilization and sterilization. The efficacy of the IM nail was evaluated in a rat femur bone transport model, and the healing process was monitored by X-ray imaging. After 34 or 55 days of operation, the specimens were harvested for assessment of bone healing through microstructural analysis, compressive testing, histological and immunohistochemical analysis, and non-decalcified histomorphometric analysis. Regenerated tissue at the early phase of bone transport was used for microarray to investigate the molecular mechanism of eluting BMP-2. A preclinical bone transport sheep model was established in the metatarsus fixing with circumferential fixator to further test the translational potential of a scaled-up 3D printed IM implant fabricated by this HyTEC technique.

Results: The hydrogel layer was attached firmly to the filament after freeze-drying, showing a smooth transition from the hydrophobic filament to the hydrophilic hydrogel at the interface and robust adhesion strength. Release kinetics of BMP-2 from the freeze-dried hydrogel-loaded implant showed a sustained and dose-dependent manner over 21 days. A prolonged release of BMP-2 could be achieved by applying additional PCL coatings onto the IM implant. Early bony fusion was achieved by the eluting BMP-2 from the IM implants through accelerating bone formation and remodeling in the rat bone transport model, with no pin tract infection but tight osseointegration were observed. The conventional treatments, on the other hand, showed high proportion of docking site nonunion and pin tract infection. Distraction over the IM implant was successfully performed in the sheep bone transport model, indicating the translational relevance of this HyTEC IM implant technique.

Discussion and Conclusion: This study indicates that with this HyTEC technique, a novel surface coating technique can incorporate a broad range of BMP-2 on the surface of scaffolds and allow for a tunable sustained release kinetics. The adhesion is achieved through a combination of covalent binding of the hydrogel to the functional groups on the PCL-TCP surface and mechanical interlocking

between the hydrogel macromonomers and cracked surface of the treated PCL-TCP. This robust adhesion ensures that the dried or rehydrated hydrogel coating remains firmly attached during and after implantation. The sustained release of BMP-2 from the IM implants was found to have an anabolic effect at the leading edge of the intercalary segment and the docking site by promoting active bone formation and facilitating bony fusion upon meeting. BMP-2 released from the implants enhanced the osseointegration between pins and surrounding bone and accelerated bone healing in both docking and regenerate sites, leading to improved mechanical stability, reduced motion at the pin-bone interface, and lowered the loosening risk that can contribute to infection. In conclusion, this novel IM implant holds great promise in revolutionizing bone transport technique in the treatment of bone defects by limb salvage through accelerating bone regeneration and mitigating complications. Acknowledgement: This project is supported by Natural Science Foundation of China (82272505), National Institutes of Health grants (R01AR057837, U01AR069395, R01AR072613, R01AR074458), and Department of Defense grant (W81XWH-22-1-0189).

## Chondrocyte-Mimicking Microspheres Decorated With Chondrocyte-Derived Exosome and Membrane for Cartilage Regeneration

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**Background:** Cartilage has a limited capacity for self-repair and regeneration due to its avascular properties. Cartilage defects are difficult to be healed due to the extremely slow migration and proliferation rates of chondrocytes. To overcome these obstacles, in this study, we prepared microspheres decorated with cell membranes and exosomes derived from healthy chondrocytes as a chondrocyte-mimicking scaffold to promote host chondrocyte migration and proliferation.

**Methods:** Porous PCL microspheres were prepared using a co-flow microfluidic system. The microspheres were subsequently coated with cell membranes isolated from primary rat chondrocytes. Chondrocyte-derived exosomes were loaded onto the chondrocyte membrane-coated microspheres. Therapeutic potentials of the chondrocyte-mimicking microspheres were evaluated on chondrocytes and bone marrow-derived mesenchymal stem cells *in vitro*. Furthermore, *in vivo* cartilage regeneration capacity of the microspheres was assessed in a rat osteochondral defect model.

**Results:** We successfully prepared highly open structured microspheres with a pore size of  $59.6 \pm 11.1 \mu\text{m}$  and a particle diameter of  $454.5 \pm 5.4 \mu\text{m}$ . A thin cell membrane layer ( $192.5 \pm 105.1 \text{ nm}$ ) was formed on the surface of the microspheres. Chondrocyte-derived exosomes were loaded onto the cell membrane-coated microspheres by lipid fusion. The chondrocyte-mimicking microspheres delivered exosomes in a more sustained manner than bolus exosome delivery methods. The released exosomes from the microspheres induced chondrocyte-specific recruitment and increased chondrocyte proliferation. Bulk RNA sequencing analysis revealed that chondrocytes cultured on the microspheres significantly up-regulated the expressions of genes related to cell adhesion, ECM synthesis, and metabolism. Interestingly, chondrocyte membrane-coated microspheres, with no exosomes, induced robust ECM synthesis via integrin-mediated mechanotransduction, which led to an increase in the anabolic activity of chondrocytes as compared to bare microspheres. *In vivo* evaluation further confirmed that the chondrocyte-mimicking microspheres improved cartilage repair by increasing the host chondrocyte migration and proliferation.

**Discussion and Conclusion:** We demonstrated that the chondrocyte-mimicking microspheres could improve cartilage repair by increasing the engagement of host chondrocytes in the defect site. The cell membrane-coated surfaces provided chondrocytes a physical cue for mechano-activation, and the exosomes delivered chemical signaling cues for regulating chondrocyte homeostasis. Our current work demonstrated a promising strategy to design biomaterial scaffolds capable of regulating chondrocyte migration and proliferation to promote cartilage regeneration.

## Investigating the effects of a cytokine storm in a micro-physiological system of the microvasculature.

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Category: Enabling technologies

Background: The systemic hyperinflammation caused by the induction of a cytokine storm is a major cause of death in COVID-19 patients. This hyperinflammatory response causes endothelial cell dysfunction, and the activation of endothelial cells, induces proinflammatory gene expression, leading to localized hyper-permeability, inflammation via recruitment of inflammatory cells to injury sites, and formation of microthrombi and microvascular damage.

Methods: M1 activated macrophages that contained a majority of factors previously reported for COVID-19 related hyper-inflammation were used to generate a cytokine storm conditioned medium, which was introduced into an in vitro microvasculature-on-a-chip model. Different essential biomarkers for endothelial functionality, such as basement membrane components, junctional proteins and endothelial activation markers, were investigated.

Results: At high concentrations of M1 conditioned medium, microvascular networks were partially disintegrated. At concentrations that allowed the majority of the network to persist, a down-regulation of laminin  $\alpha 5$  and disintegration of VE-cadherin at cell borders was observed, while von Willebrand factor was enriched within the cells.

Discussion and Conclusion: The established cytokine storm promoted disintegration of vessels, increase in vascular permeability, and endothelial activation. Functional assays will focus on investigating vascular permeability, inflammatory cell adhesion and thrombus formation in the device. The model established here thus allows studying the effects of a cytokine storm on the microvasculature in high spatial-temporal resolution.

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## A granular macroporous injectable hydrogel for promoting articular cartilage regeneration

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Category: Design and Application of Biomaterials

Background: Hydrogels are widely utilized for articular cartilage regeneration. However, traditional hydrogels with nano-sized pores lead to poor cell and tissue infiltration. Furthermore, hydrogels that can stimulate each phase of cartilage regeneration to enhance cartilage repair are highly demanded.

Method: Herein, a macroporous hydrogel is fabricated by crosslinking mixed sodium alginate (SA) and hyaluronic acid (HA) solution into SA/HA micro-fibres ( $\mu$ -fibres) first and subsequently crosslinking the  $\mu$ -fibres into hydrogel blocks. Besides, exosomes derived from NR8383 cells activated by lipopolysaccharides (LPS) and stimulated with bioglass (BG) ion extracts (LPS/BG-exo) are incorporated into the hydrogel as they exhibit a strong ability to regulate inflammation homeostasis and recruit bone marrow mesenchymal stem cells (BMMSCs). Meanwhile, poly (lactic-co-glycolic acid) (PLGA) microspheres containing kartogenin (KGN) (PLGAKGN) are encapsulated within the hydrogel for inducing the chondrogenic differentiation of recruited BMMSCs when KGN is released after the LPS/BG-exo.

Results: We found that the exosomes derived from LPS activated-NR8383 cells stimulated by BG ion extracts could effectively modulate inflammation homeostasis and recruit endogenous BMMSCs while KGN encapsulated with PLGAKGN microspheres can significantly induce the chondrogenic differentiation of BMMSCs. The obtained macro-porous SA/HAexo-PLGAKGN hydrogel could sequentially release LPS/BG-exo and KGN to regulate inflammation responses, facilitate the migration of endogenous BMMSCs, and induce the chondrocyte differentiation of BMMSCs step by step. Meanwhile, the macro-porous structure of the hydrogel could benefit the infiltration of induced cells and tissues.

Discussion and Conclusion: We demonstrated that the SA/HAexo-PLGAKGN hydrogel can not only significantly improve the infiltration of tissues into the hydrogel but also sequentially deliver LPS/BG-exo and KGN to enhance the cartilage regeneration in a rat cartilage defect model. Prospectively speaking, the SA/HAexo-PLGAKGN hydrogel is a potential candidate for other tissue regeneration as the bioactive substances can be adjusted according to different purposes.

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## Multifunctional hydrogel modulates the immune microenvironment to improve allogeneic spinal cord tissue survival for complete spinal cord injury repair

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Title: Multifunctional hydrogel modulates the immune microenvironment to improve allogeneic spinal cord tissue survival for complete spinal cord injury repair

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Category: Design and Application of Biomaterials

**Background:** Transplantation of allogeneic adult spinal cord tissues (aSCTs) to replace the injured spinal cord, serves as a promising strategy in complete spinal cord injury (SCI) repair. However, in addition to allograft immune rejection, damage-associated molecular pattern (DAMP)-mediated inflammatory microenvironments greatly impair the survival and function of transplants.

**Methods:** In this study, we aimed to regulate the immune microenvironment by developing a functional hybrid gelatin and hyaluronic acid hydrogel (F-G/H) modified with cationic polymers and anti-inflammatory cytokines that can gelatinize at both ends of the aSCT to glue the grafts for perfect matching at defects.

**Results:** The F-G/H hydrogel exhibited the capacities of DAMP scavenging, sustainably released anti-inflammatory cytokines, and reduced lymphocyte accumulation, thereby modulating the immune response and enhancing the survival and function of aSCTs. When the hydrogel was used in combination with a systemic immunosuppressive drug treatment, the locomotor functions of SCI rats were significantly improved after aSCTs and F-G/H transplantation.

**Discussion and Conclusion:** The F-G/H hydrogels can modulate the immune microenvironment, especially when used in combination with an immunosuppressive drug, thus potentially improving graft transplantation outcomes. This biomaterial-based immunomodulatory strategy may provide the potential for spinal cord graft replacement for treating SCI.

**Acknowledgement:** This work was supported by grants from the National Natural Science Foundation of China (81891002, 81971178), and the Youth Innovation Promotion Association CAS (No. 2021319).

## Nano black phosphorus/graphene oxide functionalized collagen scaffold with enhanced photo-thermal and biomimetic mineralization for in situ infectious bone repair

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**INTRODUCTION:** Infective bone defect is a common clinical problem. Infection control and bone defect reconstruction are the two main missions in traditional standard treatments. Current clinical treatments, combining the systemic application of antibiotics with autograft or allograft, have been widely used in bacterial infectious bone defects. However, these treatments have several shortcomings, such as multidrug-resistant bacteria, donor site complications, and immune rejection. Therefore, developing multifunctional bone graft substitutes with excellent biocompatibility, antibacterial property, osteoconductivity, and osteoinductivity would be a treatment strategy of great clinical significance. Collagen (Col) has been widely used to develop bone repair materials due to its good biocompatibility and biodegradability. However, collagen-based materials have the disadvantages of insufficient antibacterial performance and poor osteoconductivity. Graphene oxide (GO) and black phosphorus (BP) are commonly used two-dimensional inorganic materials, which have good photo-thermal anti-bactericidal effects under near-infrared (NIR) light. At the same time, NIR light can promote degradation of BP into PO<sub>4</sub><sup>3-</sup>, which accelerates the biomineralization process and promotes osteoconductivity. Therefore, the preparation of GO/BP functionalized Col scaffold with photo-thermal antibacterial activity and in situ biomineralization ability may provide new insights into developing multifunctional bone graft substitute for the repair of infectious bone defects.

**METHODS:** In this study, nano GO/BP particles were prepared and incorporated into Col scaffolds by one-step method. The degradation of BP under 808nm NIR was characterized. Subsequently, the physical and chemical properties of Col-GO-BP were characterized. Moreover, the biomimetic mineralization ability, photo-thermal antibacterial performance, and cell viability were evaluated. Finally, the infectious skull defect of the rat was used to evaluate their anti-infection and bone regeneration abilities.

**OUTCOME:** In this study, nano GO/BP particles were successfully prepared. The lamellar structure was observed under the TEM. Under the 808nm NIR irradiation, BP gradually degraded into PO<sub>4</sub><sup>3-</sup>. Subsequently, the Col-GO-BP scaffold was further prepared. The porous structure could be observed under the SEM. The temperature of the Col-GO-BP scaffold could stably be elevated under NIR irradiation. The cell experiment results in vitro showed that the Col-GO-BP scaffold could effectively adsorb cells, promote cell proliferation, and enhance osteoblastic differentiation. The results of animal experiments in vivo showed that the Col-GO-BP scaffold could effectively promote the repair of infectious bone defects by effectively killing bacteria, regulating oxidative stress, and reducing inflammatory reactions.

**DISCUSSION & CONCLUSIONS:** We successfully developed GO/BP functionalized Col scaffold. The GO incorporation improved cell adhesion, proliferation, and osteogenic differentiation. Moreover, the BP not only provided a phosphorus source and nucleation sites, but also accelerated the reaction between PO<sub>4</sub><sup>3-</sup> and Ca<sup>2+</sup> to promote biomineralization. Finally, the fabricated scaffold showed a good bone regeneration effect in infective bone defects by killing the bacteria, regulating the local inflammation, and improving the oxidative stress.

## Microparticles of solidified stem cell secretome (MIPSOS) for the treatment of normal and diabetic skin wounds

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**Background:** Chronic skin wounds represent a socioeconomical burden worldwide and severely reduce the quality of life of patients. Current established treatment approaches are insufficient as they do not address the dysregulated tissue environment responsible for the impaired wound healing. We have developed a biologic based on the aggregation and co-precipitation of mesenchymal stem cell (MSC)-derived extracellular matrix (ECM) with the sulfated polyglucose polymer dextran sulfate (DxS)<sup>1</sup>, termed MIPSOS<sup>2</sup>. We hypothesized that the heparan sulfate-mimicking properties of DxS will enrich the resulting semi-synthetic material in pro-regenerative and pro-angiogenic factors, thereby enhancing its therapeutic effects.

**Methods:** MSCs were induced to synthesize ECM in the presence of DxS<sup>1</sup>, which was processed into insoluble microparticles (MIPSOS)<sup>2</sup>. These were characterized *in vitro* and their therapeutic efficacy was investigated in normal and diabetic skin wound healing in mice. Comparative proteomic analysis of MIPSOS and ECM derived from naïve MSCs (cECM), enabled the identification of factors potentially responsible for the enhanced therapeutic efficacy, including IGFBP7. MIPSOS containing and lacking IGFBP7 were compared functionally.

**Results:** The insoluble format of MIPSOS protected components from degradation and enabled its sustained release. Proteomic analysis confirmed that MIPSOS was highly enriched in pro-angiogenic factors, resulting in an enhanced pro-angiogenic bioactivity when compared to cECM. Consequently, full-thickness skin wounds treated with MIPSOS demonstrated accelerated revascularization and healing in healthy and diabetic animals, far superior to the therapeutic potential of cECM and comparable to a current clinical gold standard based on tissue-derived ECM. Knock down (KD) of IGFBP7, which was found to be highly enriched in MIPSOS as compared to cECM, revealed that while endothelial cell proliferation was not affected, endothelial sprouting and migration ability was reduced on MIPSOS lacking IGFBP7. In accordance, the wound closure rate was delayed in wounds receiving MIPSOS lacking IGFBP7 with decreased blood vessel density in the wound tissue.

**Discussion and Conclusion:** We have developed an off-the-shelf biologic based on cell-derived ECM, termed MIPSOS, which is able to address major limitations of established and experimental therapeutic approaches including growth factor-, cell- and tissue ECM-based therapies. Paving the way for its clinical application, we have identified IGFBP7 as one of the responsible bioactive factors, which can be utilized as a quality control biomarker in the future. Prior research on IGFBP7 reported strongly opposing results on its pro-angiogenic properties and it was suggested that its activity may depend on the regional composition of its ECM microenvironment. Our current data suggest that within the ECM environment of MIPSOS IGFBP7 has pro-angiogenic properties promoting endothelial migration and sprouting.

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## Global transcriptome profiling of mechanoresponsive 3D bioprinted bone organoids

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**Background:** The advancements in organoid technology have expanded the possibilities for clinical drug testing and therapeutic strategy development in diverse diseases. Organoids, which are 3D structures resembling tissue and cellular architecture, can be sustained over a long period while maintaining genetic stability. They are derived from various tissues, stem cells, and even different species. Previous studies have indicated that mechanical loading is a prerequisite for forming bone organoids from human bone stem cells [1]. Additionally, it has been shown that mechanical loading plays a significant role in both the healing and remodelling of bones in in vivo studies on mouse bone [2]. However, the effect of mechanical loading on bone organoid formation and how gene expression responds to mechanical loading is unknown. Our aim is to identify the global gene expression patterns and signalling pathways activated or repressed in response to mechanical loading compared with unloading. Stem cell-derived organoids are a powerful tool for investigating molecular processes in both healthy and diseased states, and studying the gene expression profile of mechanically simulated organoids can deepen our understanding of the influence of mechanical forces on organ development and function.

**Methods:** This study created osteocyte bone organoids by 3D printing mesenchymal stem cells in a bioink composed of alginate, gelatin and graphene oxide. The organoids were subjected to mechanical loading at a frequency of 5 Hz for a pre-culture period of 4 weeks. The effect of different mechanical stimuli on the organoids was investigated by subjecting them to mechanical unloading and continued loading at 5 Hz and 10 Hz for two weeks and was compared with unloaded static cultures. The resulting changes in organoid stiffness, mineral density and bone volume were measured. Further, the RNA samples are extracted from the organoids before and after mechanical loading (10Hz) and performed RNA sequencing. The RNA sequencing data analysis were performed with quality assessment of raw sequence data and alignment to reference human genome followed by differentially expressed genes, pathways enrichment, and downstream analysis (see workflow Fig1-A).

**Results:** To investigate the mechanical properties of the organoid, the organoids were cultured in dynamic compression bioreactors, and the mechanical properties were measured using a mechanical stimulation unit. Our results show that loading organoids at a frequency of 10 Hz increased stiffness by 40% compared to unloading conditions, accompanied by a rise in tissue mineral density (Fig1-B). To examine the effects of mechanical loading and unloading on gene expression, we performed RNA sequencing and identified 173 differentially expressed genes between loading (10Hz) and unloading (Fig1-C). Further, over-representation analysis (adjusted p-value<0.05) (Fig1-D) of these differentially regulated genes revealed significant upregulation of Focal adhesion, Apelin signalling pathway, Notch signalling pathway, and cGMP-PKG signalling pathway in organoids subjected to mechanical loading, with upregulated genes such as ADAMTS6, COL4A1, COL4A2, and CLCF1.

**Discussion and Conclusion:** This study discovered that bone organoids derived from human stem cells could accurately replicate the bone niche's response to mechanical stimuli. Additionally, these bone organoids showed increased stiffness and mineralisation upon mechanical loading. The analysis of RNA sequencing data showed upregulation of the Focal Adhesion pathway and Notch signalling pathway, which is known to play a crucial role in osteogenesis and bone formation in response to mechanical loading. This finding is consistent with previous studies [3]. Additionally, we have observed the upregulation of the Apelin signalling pathway, which is known to promote osteoblast differentiation. Further, our analysis found that ADAMTS6 is a significantly upregulated gene. An earlier report has indicated that ADAMTS6 binds to the extracellular matrix structural

macromolecules fibrillin and fibronectin. This interaction has been shown to regulate focal adhesions, promoting osteogenesis [4]. In summary, our findings suggest that the Focal Adhesion, Notch, and Apelin signalling pathways, along with the ADAMTS6 gene, play a significant role in promoting the stiffness and mineralisation of bone organoids as well as osteogenesis and bone formation under mechanical loading. This study can enhance the efficacy of bone organoid models in future research, including drug screening and development.

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## Elimination of senescent BMSCs promotes bone formation and regeneration in the elderly

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**Background:** Due to the cellular senescence and declined function of bone marrow mesenchymal stem cells (BMSCs), the bone formation and repair of bone defects in the elderly is hindered. Elimination of senescent cells emerges as a promising strategy for treating age-related diseases. However, whether the elimination of senescent BMSCs can promote bone formation and regeneration in the elderly remains elusive.

**Methods:** In this study, we first screened specific senolytics for BMSCs and confirmed their ability to eliminate senescent BMSCs in vitro. We then designed a microenvironment-responsive hydrogel based on the Matrix metalloproteinases (MMPs) secreted by senescent cells to locally eliminate senescent BMSCs in bone defect. Finally, we constructed liposomes decorated with bone affinity peptide (DSS)(6) for bone-targeted delivery of quercetin to systemically eliminate senescent BMSCs in the skeleton.

**Results:** Treatment with quercetin, determined to be the best senolytic for senescent BMSCs, efficiently removed senescent cells in the BMSC population of aged individuals. The self-renewal capacity and osteogenic ability of BMSCs were restored after treatment. The quercetin-encapsulated hydrogel exhibited a stable microstructure and responsively released quercetin in the presence of senescent cells in vitro. In vivo, the quercetin-loaded hydrogel effectively cleared the local senescent cells and reduced MMP secretion in the bone of aged rodents. Due to the removal of local senescent cells, the hydrogel significantly accelerated the repair of bone defects in the femur and skull of old rats. Moreover, after administration of liposomes loaded with quercetin in two aged mouse models, histological and cellular analysis confirmed that bone-targeted treatment with quercetin efficiently eliminated senescent cells in bone, restored the function of BMCSs, and promoted bone formation in aged mouse models.

**Discussion and conclusion:** Our study revealed the promising role of eliminating senescent cells in bone regeneration and formation and provided a novel therapeutic approach for bone defects and osteoporosis in aged individuals.

## A Sustainable Corneal Tissue Engineering Platform at the Interface of Artificial Intelligence and Slaughterhouse Waste

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Title: Artificial intelligence biosensing approaches for evaluating the effectiveness of corneal decellularization

Keywords: corneal stroma; decellularization; corneal scaffold; artificial intelligence biosensing

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Abstract

**Introduction:** Decellularized corneas offer a promising source of replacement grafts. Despite great success in achieving acellular scaffolds, little consensus exists regarding the quality of the decellularized extracellular matrix (ECM). Metrics used to evaluate ECM performance are study-specific, subjective, and semi-quantitative. Thus, we focused on developing computational methods to examine corneal decellularization efficacy.

**Methods:** Corneal decellularization was performed using 1% and 4% zwitterionic surfactant concentrations for 2- and 4-day periods. Conventional biochemical and blinded semi-quantitative histological assessments were compared with automated scaffold evaluations based on textual analyses of digital micrographs to examine decellularization efficacy. The gray level co-occurrence matrix (GLCM), in combination with discrete wavelet transform (DWT) analysis, generated textual parameters that were used as input data to create contemporary artificial intelligence (AI) models based on random forests and vector machine algorithms.

**Results:** Each decellularization approach supported effective DNA removal (>95%), while manual scoring of treatment groups revealed statistically significant differences in DNA removal and changes to collagen fiber structure post-decellularization. GLCM and DWT analyses illustrated significant variations in textual parameters between the native and decellularized stroma. Regarding the machine learning models, we obtained the best performance using inverse difference moment, GLCM contrast, GLCM correlation, angular second moment and homogeneity, and wavelet coefficient energies for input data. Likewise, the estimated accuracy of the random forests model was 82.67%, while the area under the receiver operating characteristics curve was approximately 0.92. The classification accuracy of the support vector machine was 80.5% which was relatively similar to random forests. However, the support vector approach had significantly lower discriminatory power in separating treated stromal ROIs from intact stromal ROIs with the area under the receiver operating characteristics curve of 0.84.

**Discussion and Conclusions:** From our investigations, we realized that exposing corneal stromal tissues to low surfactant concentrations leads to significant changes in the features of GLCM and DWT, as with conventional histological scoring. These changes indicate a substantial increase in textural uniformity and local homogeneity and a reduction of stromal co-occurrence and wavelet heterogeneity, probably due to the loss of keratocytes and their nuclei. In this study, we also demonstrate that it is possible to develop and test machine learning models based on support vector machines and random forests using GLCM and DWT as input data, which will substantially decrease evaluation time and complexity as evidenced using multiple scorers that each required at least two days to perform these assessments.

## A Generalizable Design Strategy of Cell-laden Microgel-based Biphasic Bioink with Hyperelasticity and Heterogeneous Microenvironment for Biomedical Applications

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**Introduction:** A major challenge in three-dimensional (3D) extrusion bioprinting is the limited number of bioink that fulfill the opposing requirements for printability with requisite rheological properties and for functionality with desirable microenvironment.

**Methods:** We develop a generalizable strategy for formulating a cell-laden microgel-based biphasic (MB) bioink. The MB bioink comprises two components, i.e., microgels in close-packed condition providing excellent rheological properties for extrusion bioprinting, and a hydrogel precursor that forms a second polymer network to integrate the microgels together, providing post-printing structural stability.

**Results:** MB bioink with excellent rheological properties can be easily 3D printed into a variety of complex structures with high shape fidelity. MB bioink exhibited hyperelastic behaviors and greater cyclic performance than conventional bulk hydrogel. Moreover, we successfully demonstrated that hepatocytes and endothelial cells with spatial cell patterning by using MB bioink induced the cellular reorganization and vascularization, leading to enhanced hepatic functions.

**Discussion and Conclusion:** The flexibility in the design of MB bioink with mechanical tunability, hyperelasticity and heterogeneous microenvironment opens new possibilities for 3D bioprinting in biomedical applications such as tissue engineering and soft robotics.

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## Recombinant spider silk for cardiovascular applications: biodegradable, drug eluting, and endothelial cell-specific materials

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### INTRODUCTION

Engineered recombinant spider silk possess outstanding mechanical properties, biocompatibility<sup>1,4</sup>, biodegradability<sup>5,6</sup>, and drug delivery potential<sup>7</sup>. Furthermore, it significantly reduces foreign body reactions of coated implants<sup>2</sup>. In this work, we illustrated the potential of recombinant spider silk in cardiovascular applications. Specifically, we recombinantly expressed spider silk proteins modified with the endothelial cell-specific REDV peptide<sup>3</sup>. We then illustrated that the materials exhibit specific attachment of endothelial cells, excellent haemocompatibility, enzymatic degradability, and can be used for the delivery of clinically relevant drugs.

### METHODS

**Cell selectivity and proliferation:** Endothelial cell selectivity was observed by 4 hr attachment tests of different human cell types including human umbilical cord endothelial cells (HUVEC) on protein films. HUVEC were cultivated for 7 days various silk variants to observe the proliferation behavior.

**Blood compatibility:** To assess the blood compatibility and coagulation on the coatings, spider silk coated 316L stainless steel substrates were incubated with fluorescent fibrinogen enriched fresh human whole blood for 15, 30 and 60 minutes. Blood coagulation was analyzed by fluorescence readings, confocal and scanning electron microscopy.

**Drug elution/enzymatic degradation:** Drug elution profiles of sirolimus into phosphate buffered saline were recorded over 15 days, and degradation was assessed in a physiologically relevant mixture of enzymes.

### RESULTS & DISCUSSION

All cell types exhibited poor adhesion to the unmodified recombinant spider silk. In contrast, all cell types illustrated robust attachment to the positive controls, spider silk functionalized with the RGD ligand (spider silk-RGD) and tissue culture plastic. Furthermore, human fibroblasts and smooth muscle cells showed minimal attachment to the spider silk-REDV material, while human endothelial



cells adhesion was significantly elevated compared to the unmodified silk. Long term proliferation assays further illustrated the ability of the spider silk-REDV material to promote endothelialisation. Whole blood coagulation assays showed significant blood coagulation on uncoated 316L stainless steel (a common stent material). However, all silk materials exhibited minimal blood coagulation, illustrating the haemocompatibility of these materials. SEM and confocal images further strengthened these findings.

Enzymatic degradation and drug elution assays illustrate that the spider silks can be degraded by natural processes into natural amino acids and that clinically relevant drugs can be embedded in the materials to allow local drug delivery.

## CONCLUSIONS

spider silk is an interesting new coating material for cardiovascular stents, combining low attachment of cells and blood components with the selective adhesion of endothelial cells to form a monolayer of endothelium inside the stented blood vessel to protect the implant from the environment.

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## An interference screw made using a silk fibroin-based bulk material with high content of hydroxyapatite for anterior cruciate ligament reconstruction in a rabbit model

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Title: An interference screw made using a silk fibroin-based bulk material with high content of hydroxyapatite for anterior cruciate ligament reconstruction in a rabbit model

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Category: Design and Application of Biomaterials

Background: Upgradation is still in need for the clinically applied interference screws in anterior cruciate ligament reconstruction for more reliable fixation. Silk fibroin bulk materials offer a promising opportunity for this application except lacking osteoinductivity to some extent. Here we report a novel silk-based bulk material with high content of hydroxyapatite–silk fibroin (HA–SF) hybrid particles, which is prepared via a dual-network hydrogel.

Methods: Schematic of preparation process and the structure of the SF/HA-SF composite solid materials was shown in Figure 1. (1) Preparation of HA-SF nanoparticles. (2) Centrifugation and washing to generate pure HA–SF solution. (3) Use of H<sub>2</sub>O<sub>2</sub> and HRP as cross-linkers to make SF/HA-SF hydrogels, and then ethanol to induce  $\beta$ -sheet cross-linking points. (4) Dehydration of the hydrogels to get SF/HA-SF composite solid materials. (5) Applying in the rabbit model of anterior cruciate ligament reconstruction and were assessed histologically, radiologically and biomechanically. In addition, the cytocompatibility and the effect on osteogenic differentiation of the material were investigated in vitro via rat bone marrow-derived mesenchymal stem cells.

Results: This composite bulk material possesses a compression modulus of 3.2 GPa, comparable to that of the natural compact bone, and presents satisfactory cytocompatibility and osteoinductivity in vitro when combined with the HA–SF nanoparticles particularly. This composite bulk material shaped into interference screws exhibits remarkable biomechanical properties and significant new-bone ingrowth in the host bone tunnel in a rabbit anterior cruciate ligament reconstruction (ACLR) model at 4 weeks and 12 weeks post-operatively.

Discussion and Conclusion: In summary, we developed a silk fibroin-based bulk material with up to 40 wt% hydroxyapatite-silk fibroin hybrid nanoparticles, and focused on optimizing the interfacial adhesion between silk fibroin and inorganic substances to generate homogeneous inner structures. Due to the satisfactory dispersity of HA-SF, the mechanical properties such as stiffness and failure strength of the SF/HA-SF composite surpassed those of the SF bulk material. Moreover, the compressive modulus and yield strength of SF/40% HA-SF approached that of natural compact bone. The cytocompatibility and osteoinductivity of the SF/40% HA-SF composite made it the most qualified in vitro as a potential candidate of orthopedic implant. Animal study in vivo presented that the biomechanical properties of FGTCs in the SF/40% HA-SF group were superior to those from the group without the addition of HA, which was explained by the significantly more abundant new bone growth revealed by micro-CT and the regenerated four-layer structure at the tendon–bone interface observed in histological analysis, proving that the distinguished osteoinductivity led to a better fixation effect and surgical outcome. All the findings in the present study suggested a promising clinical translation potential of this newly developed interference screw for ACLR.



## A scalable and accessible method to achieve rapid, spontaneous, and biomimetic alignment of smooth muscle cells and endothelial cells in tissue engineered vascular grafts

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### INTRODUCTION

Tissue-engineered vascular grafts (TEVGs) have emerged as a potential alternative to autologous grafts for replacing small-diameter blood vessels during bypass surgery. The axial alignment of endothelial cells (ECs) and the circumferential alignment of smooth muscle cells (SMCs) are crucial for proper functions of native blood vessels. However, achieving this biomimetic cellular alignment in TEVGs remains a formidable challenge [1]. In this study, we aim to develop TEVGs using established biomaterials and a low-cost technique that enable the rapid axial alignment of ECs and the three-dimensional circumferential alignment of SMCs within hours, without the necessity for expensive and specialized equipment like pulsatile flow bioreactors.

### METHODS

The TEVGs consist of an electrospun layer of polycaprolactone (PCL) surrounded by a cast layer of gelatin methacryloyl (GelMA). The PCL increases the mechanical strength of the graft, while the GelMA layer prevents leakage and provides a scaffold for SMCs. We developed a freezing-induced alignment technique that quickly aligns the electrospun fibres axially, thereby inducing rapid axial alignment of seeded ECs. Furthermore, we identified a novel mechanobiological property of smooth SMCs that allow them to spontaneously align circumferentially in hydrogels of intermediate stiffness, rapidly recapitulating native blood vessel structure.

### RESULTS

ECs on the treated substrates formed a confluent endothelium and exhibited a significant increase in alignment. Moreover, it was found that SMCs cultured in a cast GelMA layer with intermediate stiffness (5-12 kPa) surrounding a PCL tube could facilitate conformation of the SMC alignment to the PCL tube curvature, resulting in spontaneous circumferential alignment within hours. In contrast, both softer and stiffer GelMA hydrogels resulted in reduced SMC alignment. Additionally, our TEVGs demonstrated statistically similar tensile modulus, ultimate tensile strength, burst pressure, and suture retention strength to native blood vessels, which will facilitate implantation and clinical translation.

### DISCUSSION AND CONCLUSION

The similarity of the mechanical properties between the TEVG and NBVs is essential for the stability and safety of the graft in the body. The full endothelium in the lumen is important as the ECs play a critical role in preventing thrombosis and in regulation of nutrient transport to surrounding cells [2].

The circumferential alignment of SMCs is essential for the mechanical integrity of the blood vessels [3].

These findings represent a significant advance in tissue engineering, enabling the fabrication of TEVGs with appropriate mechanical properties that recapitulates key NBV cell structural features, within hours, using a scalable and accessible method.

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## Trachea reconstruction by mimicking spatially heterogeneous niche with immune cascade reaction

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Title: Trachea reconstruction by mimicking spatially heterogeneous niche with immune cascade reaction

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Category: Tissue Engineering and Regeneration

**Background:** Tracheal lesions affect the ventilation function of patients, and severe cases would be life-threatening. Thoracic surgeons resort to tracheal regeneration strategy when faced with long-segment tracheal lesions. While there is no functional tracheal substitute has been used clinically. One of the crucial factors is the homeostasis of the regenerative microenvironment.

**Methods:** This study builds the spatially heterogeneous multi-niche regenerated trachea for tracheal defect repair through triblock copolymer gels (TPgel). Firstly, chondrocytes are mixed with TPgel to construct a cartilage-gel system (CGS) and complete in vitro culture. In addition, IL molecules are integrated into the hydrophilic molecules of TPgel to generate an interleukin-gel system (IGS), which can sustain sustained drug release to maintain local immune microenvironment homeostasis. Subsequently, the cartilage ring constructed from the CGS was neatly arranged, and the IGS was attached to the cartilage ring and implanted in vivo for assembly with body temperature. Finally, orthotopic tracheal transplantation will be performed on the rabbit model.

**Results:** In this study, TPgel was prepared, the optimal parameters were screened, and the characterization of its biological function was explored. A biomimetic cartilage ring was constructed to investigate the role of this process in cartilage development and performance maintenance. The immune remodeling gel was constructed to explore the immune regulation function of the factor percolation network, and reveal the mechanism pathway of the immune-regeneration cascade reaction to promote the function of cartilage and vascular network. Finally, the biomimetic trachea was constructed by assembly-prevascularization, and its long-term functional maintenance and immune microenvironment changes after orthotopic transplantation were explored.

**Discussion and Conclusion:** Orthotopic tracheal transplantation was successfully achieved in the rabbit model. This scheme exhibited satisfactory survival outcomes in rabbit models. The reconstructed biomimetic trachea displayed natural-like mechanical properties, a favorable immune microenvironment, and excellent ventilation, indicating their potential for the clinical treatment of tracheal disease.

Figure 1 Schematic of TPgel components and their use for tracheal reconstruction

## The Chemistry and Biology of Collagen Hybridization

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Title: The Chemistry and Biology of Collagen Hybridization

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Category: Enabling Technologies

Background: Collagen provides mechanical and biological support for virtually all human tissues in the extracellular matrix. Its defining molecular structure, the triple-helix, could be damaged and denatured in disease and injuries.

Methods: To probe collagen damage, we have proposed, revised, and validated the concept of collagen hybridization through a series of investigations over the past decade: [1] a collagen-mimicking peptide strand may form a hybrid triple-helix with the denatured chains of natural collagen but not the intact triple-helical collagen proteins, enabling assessment of proteolytic degradation or mechanical disruption to collagen within a tissue-of-interest.[1][2][3]

Results and Discussion: In this presentation, I will describe the concept and development of collagen hybridization and introduce the decades of chemical investigations on rules underlying collagen triple-helix folding. I will also discuss the growing biomedical evidence from our recent molecular imaging studies (e.g., targeted NIR fluorescence, MRI, and PET) on collagen denaturation as a previously-overlooked extracellular matrix signature for an array of conditions involving pathological tissue remodeling (e.g., myocardial infarction, pancreatic cancer) and mechanical injuries (e.g., osteoarthritis, tendonitis).

Conclusions: Finally, I will propose a series of emerging questions regarding the chemical and biological nature of collagen denaturation and hopefully inspire new application opportunities in diagnosis, therapeutics, and regenerative medicine by targeting denatured collagen.

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## Intelligent Microneedle Patch with Prolonged Local Release of Hydrogen and Magnesium Ions for Diabetic Wound Healing

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Category: Design and Application of Biomaterials

**Background:** The population diagnosed with diabetes has seen a mounting prevalence in the past decades, and the mortality and disability concerning diabetes are experiencing an astounding increase. Diabetic foot ulcer (DFU) is one of the most common risks of complications, affecting 15–25% of patients with diabetes mellitus. Particularly, hyperglycemic microenvironment including consistent pro-inflammatory M1 macrophage polarization, deteriorating blood vessels and accumulated reactive oxygen species (ROS) resulting from diabetes mellitus would delay the wound healing process and even lead to gangrene amputation. Hydrogen (H<sub>2</sub>) which has been proved to be therapeutic effective and non-cytotoxic can reverse this pathological microenvironment. H<sub>2</sub> as an antioxidant is capable of resisting the oxidative stress posed on tissue and cells without disturbing normal metabolic oxidation or cell signaling system. However, H<sub>2</sub> is characterized by high diffusivity, low aqueous solubility and dose-dependent effect, leading to the limited therapeutic efficacy of inhalation therapy. Therefore, it is still difficult to achieve long-term release of H<sub>2</sub> for the optimal therapeutic impact.

**Methods:** In this work, we developed a poly (lactic-co-glycolic acid) (PLGA)-based microneedle patch loaded with magnesium hydride (MgH<sub>2</sub>) macroparticles (MN-MgH<sub>2</sub>) for transdermal delivery and prolonged release of H<sub>2</sub> and Mg ions (Mg<sup>2+</sup>). Meanwhile, we also tested its efficacy on treating diabetic wounds in a minimally invasive manner in vitro and in vivo.

**Results:** MgH<sub>2</sub> can store/generate a larger amount of H<sub>2</sub> and is much more stable at room temperature. Meanwhile, the usage of PLGA-based microneedle patch enables transdermal delivery of MgH<sub>2</sub> with minimal invasiveness, protects MgH<sub>2</sub> from contacting water to prolong its lifetime and allows sustainable release of MgH<sub>2</sub> into the physiological microenvironment. Additionally, the dissolved PLGA and its degradation products (lactic acid and glycolic acid) increased the local acidity and thus promote the release of H<sub>2</sub> and Mg<sup>2+</sup>. Moreover, MN-MgH<sub>2</sub> can enhance wound healing process with reduced ROS production, promoted M2 polarization, enhanced cell proliferation and migration, as well as improved angiogenesis and tissue regeneration in vitro and in vivo.

**Discussion and Conclusion:** MN-MgH<sub>2</sub> combined the therapeutic effects of Mg<sup>2+</sup> and H<sub>2</sub> and functioned in the deep layer of tissues for wound healing. The introduction of a multifunctional MN-MgH<sub>2</sub> optimized therapeutic efficacy by i) sustainable and accelerated release of Mg<sup>2+</sup> and H<sub>2</sub>; ii) ROS reduction enabled by H<sub>2</sub> therapy; iii) enhanced polarization of M2 macrophages phenotype enabled by Mg<sup>2+</sup>. The multiple functions of MN-MgH<sub>2</sub> were systematically investigated both in vitro and in vivo, and proved to be effective in anti-inflammatory, cell proliferation and migration, angiogenesis



and tissue regeneration. This MN-MgH<sub>2</sub> that integrated various therapeutic functions provides a novel approach for improving diabetic wound healing.

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## Electrical charge on ferroelectric nanocomposite membranes enhances SHED neural differentiation

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Title: Electrical charge on ferroelectric nanocomposite membranes enhances SHED neural differentiation

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Category: Tissue Engineering and Regeneration

Background: Stem cells from human exfoliated deciduous teeth (SHED) uniquely exhibit high proliferative and neurogenic potential. Charged biomaterials have been demonstrated to promote neural differentiation of stem cells, but the dose-response effect of electrical stimuli from these materials on neural differentiation of SHED remains to be elucidated.

Methods: Here, by utilizing different annealing temperatures prior to corona poling treatment, BaTiO<sub>3</sub>/P(VDF-TrFE) ferroelectric nanocomposite membranes with varying charge polarization intensity ( $d_{33} \approx 0, 4, 12$  and  $19$  pC N<sup>-1</sup>) were fabricated.

Results: Enhanced expression of neural markers, increased cell elongation and more prominent neurite outgrowths were observed with increasing surface charge of the nanocomposite membrane, thus indicating a dose-response effect of surface electrical charge on SHED neural differentiation. Further investigations of the underlying molecular mechanisms revealed that intracellular calcium influx, focal adhesion formation, FAK-ERK mechanosensing pathway and neurogenic-related ErbB signaling pathway were implicated in the enhancement of SHED neural differentiation by surface electrical charge.

Discussion and Conclusion: Both earlier and later neurogenic differentiation of SHED appear to be dose-dependently enhanced by surface charge, as manifested by changes in cell morphology, including increased cell spreading area, cell elongation and more prominent neurite outgrowths. Surface charge stimulates opening of calcium channels on the cell membrane, resulting in an increase of intracellular calcium concentration that activated the FAK-ERK mechanosensing pathway and ErbB signaling pathway, as well as induced remodeling of actin cytoskeleton. Hence, this study confirms the dose-responsive effects of biomaterial surface charge-induced SHED neural differentiation and provides preliminary insights into the underlying molecular mechanisms and signaling pathways involved.

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## Targeting IL-6/MMP13 axis of infrapatellar fat pad to ameliorate osteoarthritis in mice

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**Introduction:** There are unmet demands on R&D of innovative and effective disease-modifying treatments for osteoarthritis (OA). OA is a multifactorial disease and the role of infrapatellar fat pad (IPFP) on OA progression is investigated in bulk. Although the IPFP-derived interleukin-6 (IL-6) may induce destruction of the synovial membrane, but further illustration for OA progression remains elusive.

**Subjects and Methods:** 12-week-old male C57BL/6 mice were subjected to destabilization of the medial meniscus (DMM) surgery or sham surgery. The knee joints of mice were collected at days 0, 3, 7, and 14 post-surgeries for histological analysis. The mRNA expression levels of fibrosis, extracellular matrix, and inflammation were analyzed with real-time PCR in both articular cartilage and infrapatellar fat pad (IPFP) at days 0, 3, 7, and 14 post-surgeries. To evaluate the effect of inflamed IPFP on OA progression, IPFP was removed at day 14 post-sham or -DMM surgery. Then there were four groups, including Sham/Sham, Sham/IPFPx, DMM/Sham, and DMM/IPFPx. The gait analysis was performed by the Catwalk system. To validate the role of IL-6 in fibrosis of IPFP, healthy mice were injected with saline or recombinant mouse IL-6 protein (rmIL-6) into the subcapsular IPFP, while DMM mice were injected with saline or IL-6-neutralizing antibody (Neu Ab) into the subcapsular IPFP. The concentration of IPFP-secreted matrix metalloproteinase 13 (MMP13) in the conditioned medium with sham- or DMM-derived IPFP was measured by ELISA. To clarify the secretory regulation of IPFP-derived MMP13 on articular chondrocytes, the rmIL-6 pre-treated IPFP was transfected with siRNA Mmp13, and then co-cultured with primary chondrocytes for 2 days. Core/shell N-isopropylmethacrylamide nanogel were synthesized and modified with RGD peptide. The lyophilized RGD-Nanogel particles were resuspended in CL82198 (MMP13 inhibitor) solution to encapsulate CL82198 through a “breathing-in” method. Mice treated RGD-Nanogel/CL82198, possessing a lasting IPFP-targeting efficiency and a desired inhibitory efficiency to the expression of Mmp13, were sacrificed at day 56 post intra-IPFP injection.

**Results:** The histopathological grade and stage of DMM group were slightly higher than that of sham group at day 14 post-surgery. The mRNA expression levels of chondrocyte hypertrophic markers in the cartilage were comparable between DMM group and sham group before day 14 post-surgery. The fibrosis in IPFP predated the damage to the articular cartilage surface at day 3 post-DMM surgery. Removal of IPFP at day 14 post-DMM surgery significantly mitigated the articular cartilage destruction and improved the functional behaviors of knee joint. The expression of IL-6 peaked in IPFP at day 7 post-DMM surgery, but the mRNA expression of Mmp13 peaked at day 7 and kept until day 56. Intra-IPFP injection of rmIL-6 could induce IPFP fibrosis in otherwise healthy mice, and intra-IPFP injection of Neu Ab could compromise the fibrogenic process in DMM mice, with less expression of alpha-smooth muscle actin ( $\alpha$ SMA) and Collagen 1A1. The concentration of IPFP-secreted matrix metalloproteinase 13 (MMP13) in the conditioned medium was significantly increased under rmIL-6 treatment relative to control group. The rmIL-6 pre-treated ex vivo IPFP was transfected with siRNA Mmp13 that could revive the extracellular matrix synthesis and simultaneously arrest the matrix metalloproteinase expression in primary chondrocytes. RGD-Nanogel prolonged the retention and efficiency of CL82198 in the joint cavity. Intra-IPFP injection of RGD-Nanogel/CL82198 biologically targeted and inhibited the expression of Mmp13 in the joint cavity, thus attenuating OA progression.

**Discussion and Conclusion:** This study demonstrates that IL-6/MMP13 axis in IPFP plays a crucial role in OA progression, including articular chondrocyte hypertrophy and IPFP itself fibrosis, and reduction of IL-6 by intra-IPFP injection of Neu Ab obviates the joint degeneration. The RGD-nanogel particles designed in this study effectively attenuate the cartilage erosion and IPFP inflammation, leading to a

development of an innovative therapeutic strategy to mitigate OA progression (Fig. 1). Our proof-of-concept study suggests that the disruption of IL-6/MMP13 axis in IPFP possesses a potential for OA treatment and the RGD-Nanogel/CL82198 method offers an avenue for the development of innovative therapeutic strategy.

Figure 1. Schematic diagram of RGD-Nanogel/CL82198 for OA treatment by disturbing the signaling in infrapatellar fat pad-derived IL-6/MMP13 axis.

## Immediately implantable polysaccharide based in situ hydrogels for new bone formation in vitro and in vivo

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**Introduction:** Bioactive molecules such as graphene oxide (GO) incorporated hydrogels have received great attention and have shown excellent potential for use in the field of bone tissue engineering due to their unique osteogenic functionalities. However, current hydrogel systems are limited in their ability to provide an appropriate amount of GO and stem cells to the lesion area as well as inconvenient transplantation processes. For instance, it has only been applied using thermosensitive injectable hydrogels.

**Subjects and Methods:** To overcome this issue, we developed a GO-incorporated injectable hydrogel system by using glycol chitosan (gC) and oxidized hyaluronic acid (oHA). Chitosan (CTS) is the second most abundant natural polysaccharide and has many valuable characteristics including biodegradability and biocompatibility, HA has also shown potential for use in the biomedical engineering area. In this study, we prepared oHA to form a hydrogel through simple and facile solution mixing without using any additional chemical or radiological crosslinking.

**Results:** Through oxidation, aldehyde groups were introduced onto the HA. Blending this with gC allowed for the formation of an aqueous hydrogel matrix. Physico-chemical characterization demonstrated that the gC/oHA/GO hydrogel matrix exhibited robust mechanical properties and stability. The gC/oHA injectable hydrogel could easily modulate the GO content and had robust mechanical properties with improved stability. As an in vitro assessment, GO-incorporated injectable hydrogel exhibited very little toxicity, but showed excellent osteogenic activity. This was confirmed by both in vitro and in vivo assessments. These results showed that GO-incorporated injectable hydrogels enhanced bone tissue regeneration as compared to control injectable hydrogels.

**Discussion and Conclusion:** Therefore, our results indicate that our injectable hydrogel system could be used for delivering GO. This material may serve as an excellent tissue scaffold for use in treating bone defects. This preliminary study will pave the way for these future investigations.

## Effect of Secretome Loaded Carrageenan-Gelatin Hydrogel on Functionalization of Airway Epithelium In Vitro Model

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Category: Design and Application of Biomaterials

**Background:** In vitro airway epithelial models are crucial for modeling diseases, studying drug interactions with tissue, and developing treatments for treating airway epithelium damage. However, current models lack complexity due to an incomplete understanding of biochemical and topographical requirements, leading to a limitation in understanding the pathophysiology and mechanism of illness. To address this issue, hydrogels are commonly used as scaffolds in tissue engineering due to their ability to sustain a 3D structure and provide mechanical support for cells. Additionally, fibroblasts are utilized to secrete essential proteins, which play a crucial role in intercellular communication. Thus, this study aims to elucidate the role of topographical and biochemical cues provided by the nasal fibroblast secretome loaded onto carrageenan-gelatin hydrogel towards formation of airway epithelium with optimal mucociliary function.

**Methods:** Respiratory epithelial cells (REC) and fibroblasts isolated from human nasal turbinate were cultured and nasal fibroblast conditioned medium (NFCM) was obtained by culturing nasal fibroblasts in mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium (DMEM) as basal medium for 48 hours. Different ratios of carr/gel were used to fabricate the hydrogel, including carr/gel (0:10), (4:6), (6:4), and (8:2). The hydrogel was cross-linked with genipin, and its physicochemical and mechanical properties, such as biodegradation rate, porosity, water contact angle, gelation time, swelling studies, ultrastructure, and biocompatibility, were characterized. The NFCM was then sorbed into a hydrogel scaffold made of kappa carrageenan (carr) and bovine gelatin (gel) with a ratio of 2% carr and 5% gel. The RECs were then seeded on the hydrogel to assess the cell attachment, proliferation, migration assay, and trans-epithelial electrical resistance, to optimize the mucociliary function of in vitro airway epithelium.

**Results:** The conditioned medium was collected in batches and standardized using pooled fibroblasts from 6 human turbinate samples. The processing and concentration of the media were done using Tangential Flow Filtration (TFF) system and ultracentrifugation technique. However, data revealed that TFF machine caused a loss of protein (289.112 µg/ml) from fresh NFCM (301.655 µg/ml), while the ultracentrifugation method resulted in a higher concentration (324.861 µg/ml). The physicochemical and mechanical properties of different ratios of carr/gel scaffold hydrogel were characterized. The contact angle for all the groups was less than 90°, which shows they were hydrophilic. The C8G2Gnp0.3 group has highest percentage of porosity (27.20±1.62%) and while for the swelling studies, C6G4Gnp0.4 has highest swelling (194.77±3.17%). For the gelation time, C8G2Gnp0.4 was the fastest group to be a gel and polymerized compared to other groups. So, in this

study, the chosen groups were to be incorporated with secretome and will be tested with further airway epithelial cells in vitro.

**Discussion and Conclusion:** This study suggests that the carrageenan-gelatin hydrogel loaded with nasal fibroblast secretome has the potential as a biomaterial scaffold for the fabrication of in vitro airway epithelium layer. The hydrogel scaffold supplemented with NFCM is capable of providing the necessary growth factors and extracellular matrix proteins required to mimic the properties of native epithelial tissue. Further studies are needed on the effect of the secretomes loaded carrageenan-gelatin hydrogel on the airway epithelial cell growth, maturation, and function to optimize its potential for airway epithelium modeling applications.

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## Bioprinting of a Hepatic Tissue Model Using Human-Induced Pluripotent Stem Cell-derived Hepatocytes for Drug-Induced Hepatotoxicity Evaluation

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**Background:** 3D bioprinting technology is an effective method for exploring the biological functions of hepatocytes by building biomimetic 3D microenvironments. Various hepatic tissue models have been developed for disease modeling, drug screening, and tissue regeneration using 3D bioprinting technology. Human-induced pluripotent stem cells (hiPSCs) are a promising cell source for the generation of functional hepatocytes. When applied in the bioprinting of hepatic tissue models using proper printing techniques and bioinks, hiPSC-derived hepatocytes (hiPSC-Heps) have demonstrated well-maintained cellular phenotypes and biofunctions, therefore are favorable for constructing hepatic tissue models for drug screening and toxicological studies.

**Methods:** The hiPSC-Heps were obtained according to an optimized differentiation protocol. A 12 × 12 × 1.2 mm grid hydrogel structure was bioprinted using a bioink composed of 7.5% gelatin and 1% sodium alginate with the suspended hiPSC-Heps at a density of 2 × 10<sup>6</sup> cells/mL. We evaluated the success of cell growth, liver-specific function, and drug-induced hepatotoxicity of the 3D-printed (3DP) model compared with the conventional 2D-cultured (2D) and the non-printed sandwich-cultured (SW) models.

**Results:** The 3DP model facilitated the formation of hiPSC-Hep spheroids with higher viability and proliferation than the commonly used non-printed SW model. The hiPSC-Heps in the 3DP model exhibited higher mRNA expression of liver-specific functions than those in the 2D model. Moreover, enhanced secretion of liver function-related proteins, including  $\alpha$ -1-antitrypsin, albumin, and blood urea nitrogen, was observed in the 3DP model. For the evaluation of acetaminophen-induced hepatotoxicity, the 3DP model exhibited a favorable drug response with upregulation of the drug metabolism-related gene cytochrome P450-1A2 (CYP1A2).

**Discussion and Conclusion:** A hepatic tissue model was constructed by 3D bioprinting hiPSC-Heps using an alginate-gelatin bioink. Compared with the 2D and non-printed SW model, the hiPSC-Heps in the 3DP model exhibited favorable cell viability and proliferation, liver-related function, and drug response performance with the upregulation of the drug metabolic enzyme CYP1A2. This 3DP model could be used as an advanced hepatic tissue model for potential applications in *in vitro* toxicological studies.

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## Design of New Forms of Electrospun Nanofiber Materials for Tissue Regeneration

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Title: Design of New Forms of Electrospun Nanofiber Materials for Tissue Regeneration

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Category: Design and Application of Biomaterials

Background: Electrospun nanofibers have been emerged as biomimetic matrices for tissue modeling, repair, and regeneration in combination with therapeutics and living cells. However, the insufficient porosity, small pore size, noninjectability, and inaccurate spatial control inhibit the practical applications of traditional electrospun nanofiber materials in tissue engineering and regenerative medicine. Many efforts have been devoted to exploring new forms of nanofiber materials.

Methods: In this work, we developed a gas-foaming expansion technology for producing three-dimensional nanofiber objects with various shapes. We also developed a co-axial electro spray method to fabricate nanofiber microspheres with controlled structure and composition. We demonstrated the potential applications of these new forms of electrospun nanofiber materials in wound healing and bone regeneration.

Results: We invented a novel approach inspired by solids of revolution that transforms 2D nanofiber membranes into previously inaccessible 3D objects with customized shapes. The resultant 3D nanofiber objects with high porosity consisted of numerous layers of aligned nanofiber membranes. The gaps between adjacent layers ranged from several microns to several millimeters. Such nanofiber objects can regain their shape after release of compression. These objects can be used as scaffolds to guide the organization of cells and fabricate highly ordered 3D tissue constructs. 3D scaffolds consisting of radially aligned nanofibers guided and promoted the migration of bone marrow stem cells from the surrounding region to the center in vitro. Subcutaneous implantation of 3D nanofiber objects in rats showed rapid cell infiltration, neo vessel formation, and collagen production. In addition, these 3D nanofiber scaffolds showed the highest new bone volume, surface coverage, and mineral density among the tested group in a rat critical-sized calvaria bone defect model.

Discussion and Conclusion: We have demonstrated a novel approach to convert 2D nanofiber membranes to 3D nanofiber objects with predesigned, complex shapes. These 3D scaffolds can retain the alignment of nanofibers. We also demonstrated 3D scaffolds consisting of radially aligned nanofibers can promote cranial bone regeneration. We envision the combination of the complex 3D objects with hydrogels and 3D printing technology for engineering 3D in vitro tissue models and constructs and regenerating tissues in the near future.

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## Targeting lncRNA H19 by magnetic metal-organic frameworks to ameliorate abnormal subchondral bone remodeling and cartilage degradation in osteoarthritis

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**Background:** Targeting the perturbed subchondral bone remodeling has been proposed to minimize uneven articular cartilage stress distribution, and thus representing a new therapeutic approach for osteoarthritis (OA) [1]. A long non-coding RNA (lncRNA) H19 was reported to be associated with OA progression and regulate mechano-transduction at cellular level [2], however, its role in OA subchondral bone has not been reported before. Furthermore, it is anticipated that lncRNA antisense oligonucleotides (ASOs) could be potential candidates for treating OA [3], which is limited because of their weak localization, low retention and poor biocompatibility. This study aimed to examine the relationship between osteocytic H19 and OA subchondral bone remodeling, and to develop an advanced gene-delivery system based on metal-organic frameworks (MOFs) to target H19 in subchondral bone for OA treatment.

**Subjects and Methods:** Subchondral bone were collected from patients with knee OA undergoing joint replacement surgery (n = 12). To verify the link between H19 and OA subchondral bone, wild-type C57BL/6 mice and same background transgenic mice with osteocyte-specific deletion of H19 (cKO) mice were used, and OA phenotype was induced by DMM (destabilization of the medial meniscus) surgery. H19 expression was determined by fluorescence in situ hybridization (FISH) and qPCR, subchondral bone changes by microCT, and OA phenotypes by histological analysis. [4]. Combined with Fe<sub>3</sub>O<sub>4</sub> nanoparticles, a magnetic MOFs (MMOFs) was developed as a nanoplatform to deliver anti-H19 ASO to the target site via magnetic field for OA and subchondral bone remodeling therapy. Paired t-test was used for comparing differences in human subchondral bone. One way ANOVA and Dunnett's multiple comparisons test were used to compare differences in animal and cellular experiments.

**Results:** Human subchondral bone of end-stage OA had higher level of H19 in both femoral condyle and tibial plateaus, which is associated with increased bone mass and more H19 expressing osteocytes. In wild-type C57BL/6 mice, DMM surgery led to cartilage damage, subchondral sclerosis, and increased H19 expression in subchondral bone. On the contrary, cKO mice with H19 ablation were less vulnerable to DMM induced OA phenotypic changes. MMOFs were successfully synthesized, and shown as an efficient delivery system with good bioavailability and effectiveness. In vivo ASO@MMOFs targeting H19 substantially alleviated subchondral bone remodeling and OA phenotype.

**Discussion and Conclusion:** Our results provide new evidence that elevated H19 expression in osteocytes may contribute to the aberrant subchondral bone remodeling and thus cartilage damage in OA development. And this was the first attempt to investigate the targeted inhibition of lncRNA H19 in subchondral bone by using MMOFs, which represented a promising new approach for OA treatment.

**References:** [1] Bailey et al., 2021. [2] Wang et al., 2022. [3] Chen et al., 2016 [4] Lawson et. al., 2021

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## Assessing Biomaterial-induced Stem Cell Lineage Fate by Machine Learning-based Artificial Intelligence

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Category: Tissue Engineering and Regeneration

Introduction: Current functional assessment of biomaterial-induced stem cell lineage fate in vitro mainly relies on biomarker-dependent methods with limited accuracy and efficiency. This study aims to provide an advanced strategy to evaluate biomaterials in inducing stem cell differentiation based on transcriptomics and machine learning.

Subjects and Methods: In this work, a gene expression reference was generated by integrating public RNA-seq data related to tri-lineage differentiation (osteogenesis, chondrogenesis, and adipogenesis) of human mesenchymal stem cells (hMSCs). And an intelligent assessment model was established using algorithms containing the k-nearest neighbors (kNN) strategy and then evaluated using both an external testing dataset and multiple biomaterials.

Results: A framework named “Mesenchymal stem cell Differentiation Prediction (MeD-P)” was reported for biomaterial-induced cell lineage fate prediction. MeD-P contains a cell-type-specific gene expression profile as a reference and a predictive model for classifying hMSCs differentiation lineages. MeD-P exhibits an overall accuracy of 90.63% on testing dataset, which is significantly higher than the model constructed based on canonical marker genes (80.21%). Moreover, evaluations of multiple biomaterials show that MeD-P provides accurate prediction of lineage fate on different types of biomaterials as early as the first week of hMSCs culture.

Discussion and Conclusion: We demonstrated that MeD-P was trained to be robust and accurate with large-scale public RNA-seq data after correcting for batch variances. MeD-P is an efficient and accurate strategy for stem cell lineage fate prediction and standardized functional biomaterial evaluation. It demonstrates much potential in facilitating biological performance optimization of biomaterials for tissue regenerative therapies.

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## Magnesium-containing dressings promote deep wound healing via activating fascia mobilization and neurovascular interaction

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**Background:** Deep wounds involving subcutaneous tissue are hard to repair, especially for diabetic deep wounds with chronic inflammation, vasculopathy and neuropathy. The subcutaneous fascia was first reported for acute deep wounds repair in the top journal, *Nature* [1], and our previous works reported that Mg<sup>2+</sup> ions could stimulate periosteum to improve bone-fracture healing [2] and induce peripheral nerve regeneration [3]. However, the effect of fascia on chronic wounds is far from clear and whether Mg<sup>2+</sup> can be used to stimulate fascia to modulate the wound healing process remains to be disclosed. In addition, the effect of Mg<sup>2+</sup> on neurovascular interaction during this process deserves to be explored.

**Methods:** Herein, Mg containing sprays were synthesized by a microwave-assisted hydrothermal reaction, and further used to load anti-inflammatory drug curcumin (Cur) and modified with Mg<sup>2+</sup>-dependent oligo sequences to achieve a controllable release. Diabetic rats were induced with high-fat and streptozotocin (35 mg/kg). For in situ fascia tracing, FITC NHS dye at 10 mg/mL in saline was subcutaneously injected (50 µL for each wound) at four and two days before modelling. Then, deep skin wound model was made according to previous report [1] and treated with prepared sprays for 14 days. For immunolabelling, the sections were incubated with primary antibody to CD31 (AF3628), α-SMA (MA5-11547), and TUBB3 (ab18207), and then mounted with DAPI for imaging. HUVECs and Schwann cells (SCs) were co-cultured in vitro to explore the effect of Mg<sup>2+</sup> on neurovascular interaction.

**Results:** Bioactive MgSiO<sub>3</sub> (MgSi) sprays were synthesized with amorphous phase, nanoscale, and high specific surface area, which revealed a pH-responsive degradability and effective drug loading capacity (Fig.1A). Then, Oligo@Cur@MgSi sprays were successfully prepared (Fig.1B). Here, the advanced Oligo caps kept turn-off in stock to prevent Cur leaking, and then responsively transferred to a turn-on state with the activation of Mg<sup>2+</sup> ions for controlled Cur releasing (Fig.1C). The key of Mg<sup>2+</sup> ions was supplied by MgSi degradation, and its quantity could be amplified by an acidic pH niche (pH < 7). For deep wound healing, bioactive MgSi could activate the mobilization of fascia from the bottom of adjacent tissue upwards the center and surface of wound, which facilitated the healing rate and stimulated the regeneration of blood vessels and peripheral nerves (Fig.1D-E). Additionally, MgSi promoted the neurovascular interaction, concretely, the released ions could stimulate SCs to secrete CGRP, which revealed a promoting effect on HUVECs proliferation and new vessels formation. In turn, the secreted VEGF of HUVECs also promoted neurites outgrowth and peripheral nerves regeneration (Fig.1F). While for Oligo@Cur@MgSi sprays, they could initially reprogram macrophage to a pro-healing M2 phenotype to effectively inhibit the severe inflammation caused by diabetes, and then significantly reactivated fascia mobilization and neurovascular interaction during the proliferation stage to achieve a chronologically accelerated healing process.

**Discussion and Conclusion:** Considering the persistent inflammatory storm, reduced cell viability and severe depth of diabetic deep skin wound in clinic, in this work, we validated the promising effect and mechanism of Oligo@Cur@MgSi sprays on diabetic deep wound healing. Under the direction of a whole-course-repair therapeutic strategy, chronic inflammation was first reversed, and then proliferation and remodelling phase were accelerated via fascia mobilization and neurovascular interaction activated by the released bioactive ions (Mg<sup>2+</sup>, SiO<sub>3</sub><sup>2-</sup>). Taken together, Mg-containing dressing may be a feasible option for future deep wound management.

**Acknowledgement:** This work was supported by Areas of Excellence from the Research Grants Council of Hong Kong (AoE/M-402/20). Reference: [1] Correa-Gallegos, et al. *Nature* 576 (2019) 287-292. [2] Zhang, et al. *Nat. Med.* 22 (2016) 1160-1169. [3] Yao, et al. *Adv. Sci.* 9 (2022) 2202102.

## 3D-printed scaffold with halloysite nanotubes laden as a sequential drug delivery system regulates vascularized bone tissue healing

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Title: 3D-printed scaffold with halloysite nanotubes laden as a sequential drug delivery system regulates vascularized bone tissue healing

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Category: SYIS plus Tissue Engineering and Regeneration

**Introduction:** Bone repair is a complex, multi-stage process, involving angiogenesis and osteogenesis, under the control of sequential stimulation from multiple factors such as FGFs, VEGF, and BMP2. Promoting the bone repair processes at different stages can greatly shorten the period and improve the quality of repair. How to deliver angiogenic and osteogenic cues in sequence still remains a challenge. Current study highlights the great potential of scaffold with a sequential delivery platform for clinical application.

**Subjects and Methods:** This research proposed a 3D-printed scaffold with a sequential delivery platform loaded with nanotubes and microspheres to realize the coupling regeneration of blood vessels and bones. Deferoxamine was loaded onto halloysite nanotubes by electrostatic interaction to promote the pre-vascularization of the defect area, which can provide a better blood supply for the subsequent regeneration of bone tissue. BMP2 was encapsulated into the microspheres to achieve continuous long-term osteogenic induction. The PLGA/TCP solution mixed with microspheres and halloysite nanotubes was shaped into a scaffold by 3D low-temperature deposition printing to ensure drug inactivation did not occur.

**Results:** The drug delivery system inside the scaffold released pro-angiogenic drugs within the first week of repair and maintained an effective level of bone regeneration-promoting growth factor for an extended period, consistent with the cascade of the natural bone repair process.

**Discussion and Conclusion:** A 3D-printed scaffold with a sequential drug delivery system laden for the regulation of vascularized bone tissue healing was proposed and fabricated through low-temperature deposition 3D printing technology. The vascular-promoting drug DFO and the bone-promoting growth factor BMP2 were loaded onto electric nanoparticles and into microspheres, respectively, following different release kinetics to meet the needs of different bone repair stages. Such a PLGA+TCP+HNT+MS scaffold with sequential drug delivery concept could contribute to the treatment of large-segment bone defects in clinical trial.

## Systemic Administration of High Mobility Group Box 1 Fragment Improves Cardiac Functions by Activating a Tissue Healing Pathway of Bone-Marrow Mesenchymal Stem Cell in a Porcine Ischemic Cardiomyopathy Model

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**Introduction:** Ischemic cardiomyopathy (ICM) is a disease with a poor prognosis, and many patients undergo left ventricular assisted device (LVAD) implantation or heart transplantation. Regenerative therapy is a topic of interest for treating severe heart failure. High mobility group box 1 (HMGB1) is a nuclear protein that has many roles in maintaining homeostasis as a regulator of transcription, a mediator of inflammation, or a factor related to tissue repair. It has been reported that HMGB1 fragment (a domain of HMGB1 protein) mobilizes bone marrow mesenchymal stem cells (BM-MSC) from bone marrow (BM) to the injured tissue in a myocardial infarction (MI) rat model. We evaluated the effectiveness of the HMGB1 fragment using various imaging modalities in a MI model porcine and positioned this research as translational research for the clinical application of the HMGB1 fragment.

**Methods:** We used female Gottingen mini pigs weighing 15-25kg. An ICM model porcine was generated by placing an ameroid constrictor ring (internal diameter: 3.5mm) on the ostium of the left anterior descending artery (LAD). After 4 weeks, HMGB1 fragment (3mg/kg) or normal saline was administered intravenously over 10-15 minutes, 5 times, every 2 days. In the control group, we administered the same volume of normal saline as the HMGB1 fragment group. We divided the model into two groups (HMGB1 fragment group and control group) and performed echocardiography, cardiac magnetic resonance imaging (cMRI), and pressure wire study. Echocardiography was performed at 4 weeks and 8 weeks after treatment, and the other modalities were performed at 8 weeks after treatment. After that, the porcines were euthanized, and histological analysis and real-time PCR (RT-PCR) were performed.

**Results:** In echocardiography, the ejection fraction (EF) was improved (HMGB1 vs control;  $\Delta$ EF +11.1% vs -4.8%,  $p=0.0015$ ), and end-systolic volume (ESV) became smaller (HMGB1 vs control;  $\Delta$ ESV -1.5ml vs +7.3ml,  $p=0.0043$ ) in the HMGB1 group. In cMRI, volumetry showed the same results as echocardiography (HMGB1 vs control;  $\Delta$ EF +7.3% vs -4.5%,  $p=0.0090$ ). Regional left ventricular strain was improved in the HMGB1 group, not only in the LAD region but also in the left circumflex or right coronary artery region. Late gadolinium enhancement (LGE) showed that the damaged myocardium zone (enhanced area) became smaller in the HMGB1 group (HMGB1 vs control;  $\Delta$ enhanced area rate at LAD lesion -11.5% vs +17.9%,  $p=0.0062$ ). In the pressure wire study, we calculated the coronary flow reserve (CFR) and resistive reserve ratio (RRR). They were improved in the HMGB1 group (HMGB1 vs control;  $\Delta$ CFR +0.45 vs +0.01,  $p=0.0166$ ,  $\Delta$ RRR +0.50 vs +0.07,  $p=0.0176$ ). In the histological analysis, the cardiomyocyte diameter was smaller with hematoxylin-eosin stain, the area of fibrosis was smaller with Masson-trichrome stain, and there were more CD31 positive endothelial cells detected in the HMGB1 group. In RT-PCR analysis, the levels of factors for angiogenesis, anti-fibrosis, and anti-inflammation, such as HGF, FGF2, SDF1, TGF $\beta$ 3, and IL-10, were higher in the HMGB1 group.

**Discussion and Conclusion:** HMGB1 fragment mobilizes BM-MSCs to the damaged myocardium, which then secrete various cytokines and promote angiogenesis, anti-fibrosis, and anti-apoptosis,

and regulate inflammation. This induces improvement of coronary flow, vascular function, and myocardial damage, resulting in tissue repair and improved cardiac function. As the HMGB1 fragment can be intravenously administered immediately after thawing, it is expected to have tissue regeneration inducing effects with low invasiveness and high versatility. This is a potential drug for a new treatment option for severe heart failure. The HMGB1 fragment, which induced mobilization of MSCs from the bone marrow to the peripheral blood and directed them to the injured area, improved cardiac function in an ICM porcine model by promoting angiogenesis and inhibiting fibrosis.

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## Differentiation of RPE Cells from Human Pluripotent Stem Cells in Xeno-free Conditions

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**Introduction:** Retinal degenerative diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (PR), are the leading causes of irreversible blindness in human. The cell death or dysfunction of retinal pigment epithelium (RPE) cells often leads to accumulation and apoptosis of photoreceptor cells, thus causing patients' vision loss. Because the dead RPE cells are difficult to be regenerated, most of the existing therapeutic methods, especially for patients with advanced dry AMD, do not show a good improvement of the vision loss. Subretinal transplantation of RPE cells derived from hPSCs are the potential cell replacement therapy for retinal degenerative diseases. However, the current method of differentiating hPSCs into RPE cells is time-consuming and inefficient, and the development of xeno-free RPE cells cultured on specific biomaterials is urgent for future clinical applications.<sup>1</sup> In this study, we compared different protocols and cell culture biomaterials coated with extracellular matrix (ECM) proteins and investigated the effectiveness and robustness of hPSC differentiation into RPE cells cultured on cell culture biomaterials. We hope to design and develop an effective protocol for hPSC differentiation into RPE cells for future clinical trials.

**Subjects and Methods:** hPSCs were differentiated into RPE cells by two RPE cell differentiation protocols using small molecules and/or proteins (Fig. 1).<sup>2,3</sup> We also compared the differentiation efficiency of RPE cells in dishes coated with Matrigel, laminin511 (LN511), laminin521 (LN521), and human recombinant vitronectin (rVN). Immunostaining and flow cytometry were performed to detect the expression of RPE cells related surface markers. After 84 days of differentiation, hPSC-RPE cell suspension that is stained with CellTracker Red was transplanted into Royal College of Surgeons rats (RCS, AMD disease model rats) at 21 days of the rat age. Full field electroretinogram (ERG) was recorded after dark adaptation of 1 month and 2 months after transplantation.

**Results:** The pigmented cells of hPSCs-derived RPE cells were successfully generated from hPSCs using activin A and NIC84 protocols. The cells showed a hexagonal tightly connected "cobble stone" structure from phase contrast microscopy observation. hPSCs-derived RPE cells showed strong expression of RPE functional cell markers (PAX6, ZO1, RPE65, and MITF) using immunostaining method. The functional hPSC-RPE cells were successfully prepared by using these two chemical defined protocols. The expression ratio of neural marker (PAX6), small eye transcription factor (MITF), mature RPE marker (RPE65) and tight junction protein (ZO1) were evaluated using flow cytometry. The survival ratio of hPSCs-derived-RPE cells, which were cultured on different ECM-coated dishes were used to investigate the efficiency of hPSC differentiation into RPE cells. Fundus imaging after cell transplantation in RCS rats showed that hPSC-derived RPE cells were successfully transplanted under the neural retina. ERG results showed that the b wave amplitude of the eyes transplanted with hPSC-derived RPE was higher than that of the control group at one month after transplantation, indicating some vision improvement of RCS rats by transplantation of hPSC-derived RPE cells.

**Discussion and Conclusion:** hPSCs could be cultured in a culture dish with chemically definite medium and maintain high pluripotency and proliferation ability. In this study, hPSCs were successfully induced to differentiate into RPE cells by modifying two RPE differentiation protocols (Activin A and NIC84). The transplantation of differentiated RPE cells into RCS rats could help to delay vision loss in RCS rats. We also investigated the differentiation efficiency of RPE cells, which were cultured on rVN, LN511 and LN521 coated dishes. From the expression of RPE markers (PAX6, ZO-1, MITF, RPE65) cultured on ECM-coated dishes, LN521-coated dishes have benefits to induce the efficient differentiation of hPSCs into RPE in xeno-free conditions.

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## Multi-functional Tannic Acid-Magnesium Nanoparticles for Osteoimmunomodulation and Vascularization of Bone Defects

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**Background:** Formal bone tissue regeneration studies focused mainly on the differentiation of stem cells and vascularization. However, as the role of the immune system in tissue regeneration became more evident, many attempts have been made to manipulate these immune cells. ROS-scavenging materials have shown potential for creating a regeneration-friendly immune environment by increasing the anti-inflammatory M2 macrophages. One such material is tannic acid(TA), a naturally occurring polyphenol. In this study, magnesium ions were composited with TA via the metal-phenolic network(MPN) to allow enhancement of stem cell differentiation and vascularization.

**Method:** In this study, tannic acid-magnesium nanoparticles(TMgP) were fabricated via a metal-phenolic network. The particles were incorporated into gelatin-based cryogels for delivery. These composite cryogels were used to test their effects on immunomodulation, angiogenesis and osteogenesis both in vitro and in vivo.

**Results:** The PCR and IHC results showed an increase in M2 markers and a decrease in M1 markers in RAW264.7 cells when treated with G-TMgP extracts. Also, a reduction in TRACP activity was noticed in TMgP-treated groups. A higher expression level of osteogenic genes and proteins in adipose-derived stem cells (ADSCs) cultured with G-TMgP extracts was confirmed. Human umbilical vein endothelial cells (HUVECs) could maintain their tubular structure longer and migrate better after TMgP treatment. ADSCs also showed increased angiogenic cytokine expression levels. In the mouse calvarial defect model, gels with higher concentrations of TMgP showed higher levels of bone recovery. Furthermore, a higher number of vessels were present in the regenerated bones treated with higher concentrations of TMgPs.

**Discussion and Conclusion:** This study confirmed that the TMgPs were able to modulate the immune environment by increasing the M2 portion of macrophages. Also, osteogenic differentiation and angiogenesis were confirmed in vitro, which led to higher bone regeneration and vascularization levels in vivo. This concludes that TMgPs were able to enhance bone regeneration by creating a regeneration-friendly immune environment and inducing osteogenic and angiogenic abilities of cells.

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## Design and validation of performance-oriented injectable chitosan thermosensitive hydrogels for endoscopic submucosal dissection

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Category: Design and Application of Biomaterials

**Background:** Endoscopic submucosal dissection (ESD) is a well-established, minimally invasive treatment for premalignant and early malignant gastrointestinal lesions. A liquid cushion is created by submucosal injection to lift and separate the lesion and malignant portion from the muscular layer and create a space endoscopic incision, an essential step in ESD. Currently, many intraoperative biomaterials used in ESD are being studied, and their designs have been considered from different perspectives. However, the use of biomaterials to improve the operator's convenience (operating affinity) has received little attention. From the perspective of clinical operation, how to design and optimize the hydrogel system used in ESD is worth discussing.

**Methods:** In this work, we prepared two thermosensitive hydrogels, lactobionic acid-modified chitosan/chitosan/ $\beta$ -glycerophosphate thermosensitive hydrogel (hydrogel 1) and its lyophilized powders (hydrogel 2), characterized their physicochemical properties and evaluated their performance in ESD experiments on large animals, by comparing with the commonly used normal saline (NS) and glycerin fructose (GF).

**Results:** The two hydrogels showed good low-temperature fluidity. The hydrogels provided significantly better viscoelastic properties than NS and GF. The hydrogels can be maintained for seven days, even at pH 1, after which they degrade entirely. In pig model experiments, we performed submucosal injection and ESD procedures in the stomach and esophagus. The cushion height produced by the hydrogels was higher than those of NS and GF 30 min after injection. The ESD operation time for hydrogels was significantly shorter. Postoperative wound observation and histological analysis showed that the hydrogels promoted wound healing.

**Discussion and Conclusion:** Lactobionic acid-modified chitosan/chitosan/ $\beta$ -glycerophosphate thermosensitive hydrogel and its lyophilized powders hydrogel showed excellent performance. They shorten operation time and promote wound healing after ESD. The two hydrogels showed differences in performance and could be selected according to the size and location of the lesion during ESD.

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## Fabrication of 3D vascularized fat tissue using co-culture spheroid-laden 3D PCL scaffold with GelMA hydrogel

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Title: Fabrication of 3D vascularized fat tissue using co-culture spheroid-laden 3D PCL scaffold with GelMA hydrogel

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Category: Tissue Engineering and Regeneration

Background: Soft tissue augmentation is commonly achieved through the use of enriched grafts or liposuctioned tissue. However, these methods have limitations, including donor site morbidity, limited yield, and the potential for long-term capsular contracture. Adipose tissue engineering has been proposed as an alternative solution, but previous attempts to fabricate vascularized fat tissue by combining cells and biomaterials have been hindered by the toxicity of adipogenic differentiation inducible culture conditions for endothelial cells.

Methods: In this study, we developed a novel adipo-inducible nanofiber that automatically triggers adipogenic differentiation in human adipose-derived stem cell (hADSC) spheroids. The spheroids were then positioned in a 3D polycaprolactone (PCL) scaffold and mixed with GelMA hydrogel to enable the fabrication of 3D vascularized fat tissue capable of long-term culture.

Results: A co-culture spheroid of hADSCs and HUVECs with a uniform diameter was successfully fabricated. The spheroid demonstrated enhanced sprouting in GelMA hydrogel when positioned on the 3D PCL scaffold compared to a suspension of single cells. Directional control of sprouting cells was observed along the strands of the 3D PCL scaffold, resulting in improved ECM remodeling and angiogenic gene expression. This engineered system maintained its shape for 28 days of culture and induced adipogenic differentiation of hADSCs by adipo-inductive nanofibers without differentiation medium, leading to the formation of lipid droplets and improved adipogenic gene expression.

Discussion and Conclusion: The study findings indicate that spheroids with enhanced cell-cell interaction have a greater potential for ECM remodeling compared to single cell suspensions and exhibit improved sprouting within the hydrogel. Additionally, the directional control of sprouting along the strands of the 3D PCL scaffold enhances vascularization, as evidenced by the improved vascularization of endothelial cells when direction is controlled through an existing reference. The successful formation of 3D vascularized fat tissue was demonstrated through the use of adipo-inductive nanofibers to induce adipogenic differentiation of stem cells in spheroids and promote simultaneous vascularization of endothelial cells, even without the use of a differentiation medium. These findings hold significant potential for developing clinically relevant adipose tissue engineering techniques with improved vascularization.

Acknowledgement: This research was supported by National Research Foundation of Korea (NRF) grants funded by the Korean government (MEST) (NRF-2020M3H4A1A02084829).

## Stem cell encapsulated adhesive protein-based complex coacervate for stem cell transplantation for cartilage reconstruction

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### Background:

Articular cartilage (AC) is the smooth tissue covering the bones' end and absorbing external impacts. AC can be damaged by various types of impacts. Thus, damaged AC can trigger the development of osteoarthritis (OA). With the limited intrinsic healing capacity of AC, cell transplantation has been recognized as a possible means of AC reconstitution. However, transplanted stem cells disappear from the transplantation site rapidly, abrogating the original intent of cell transplantation. In the present work, a human adipose stem cell (hASC)-encapsulated mussel adhesive protein-hyaluronic acid (MAP-HA) coacervate was applied to extend the retention of transplanted stem cells and improve cartilage regeneration.

### Methods:

In this research, we construct the MAP-HA coacervate by manipulating the molecular weight (MW) and blending ratio of HA. Rheological and underwater adhesive analyses were conducted to identify the optimal MAP-HA composition for AC environment applications. Cytocompatibility analyses were performed with hASCs to confirm the coacervate's safety for stem cell encapsulation. An in vivo rabbit model of combined OA and chondral defects was employed to assess the therapeutic efficacy of the hASC encapsulated MAP-HA coacervate in comparison to fibrin glue.

### Results:

This study revealed that the MAP-HA723 coacervate possessed appropriate properties for AC environment applications, such as underwater adhesion and suitable viscosity. The coacervate displayed cytocompatibility with hASCs, allowing for their survival without interfering with their functionality. In the in vivo rabbit model, the MAP-HA723 coacervate facilitated better retention of transplanted hASCs on chondral defects, resulting in enhanced regeneration of damaged AC compared to fibrin glue.

### Discussion and Conclusion:

In this study, we developed hASC encapsulated MAP-HA coacervate to overcome the limitations of traditional cell therapy for cartilage defect and OA. Developed MAP-HA coacervate a viscous immiscible liquid-phase protein bioadhesive, as an injectable surgical method that enables prolonged retention of transplanted stem cells for effective reconstitution of chondral defects. We were able to form a complex coacervate with properties suitable for AC environment application by controlling the molecular weight and blending ratio of HA. Through in vivo rabbit model evaluation, we successfully demonstrated that MAP-HA723 coacervate can retain and prolong the survival of transplanted hASCs on chondral defects and thereby better regenerate the damaged AC compared with the application of fibrin glue. Collectively, the developed viscous immiscible liquid-phase protein bioadhesive can provide a suitable adjunctive option for the effective application of cell therapy to regenerate AC.

## Stem cell-derived exosomes regulate endogenous neural stem cells and delivery drug to promote nerve regeneration

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Title: Stem cell-derived exosomes regulate endogenous neural stem cells and delivery drug to promote nerve regeneration

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Category: Tissue Engineering and Regeneration

**Background:** Spinal cord injury (SCI) commonly disrupts the neuronal connections between the brain and the periphery, leading to temporary or permanent loss of sensory and motor functions. Based on tissue engineering technology, there recently emerges a promising way by integrating drugs with suitable scaffold biomaterials to mediate endogenous neural stem cells (NSCs) to achieve one-step SCI repair. As nanoscale vesicles, exosomes not only have their own biological activity, but also serve as drug delivery vehicles. Therefore, exploring the effect of exosomes on neural stem cells and simultaneously using them to deliver drugs, that is, to kill two birds with one stone, demonstrate the potential for the treatment of SCI. However, how to realize the effective combination of exosomes and scaffold materials is a key problem.

**Methods:** We chose the exosomes derived from NSCs and mesenchymal stem cells, respectively. We verified the effects of stem cell-derived exosomes on migration, proliferation and differentiation fate of NSCs by CCK8, immunofluorescence staining, and transwell, respectively. The dual bio-specificity peptide BSP was synthesized in chemical solid-phase peptide synthesis. One end of it was the collagen-binding domain, and another end was a 7-peptide that specifically binds to transferrin on exosomes. We applied both confocal microscopy and scanning electron microscopy to prove that BSP can effectively link exosomes with collagen materials. Furthermore, we chose a clinically approved PTX as the exosome-loaded model drug, resulting in a functional collagen scaffold. Completely transected SCI rats were used to verify the therapeutic effect of the functional collagen scaffold by virtue of mediating endogenous NSCs.

**Results:** We found NSCs-derived exosomes (NExos) promote the proliferation of NSCs by regulating the MEK/ERK/CREB signaling pathway, and mesenchymal stem cell-derived exosomes (MExos) can promote the migration of NSCs both in vivo and in vitro. By virtue of the synergy that exosomes mediate endogenous NSCs, and PTX induce NSCs to give rise to neurons, the multifunctional collagen scaffolds have shown superior performance for motor functional recovery after complete SCI in rats by enhancing neural regeneration and reducing scar deposition.

**Discussion and Conclusion:**

In the context of SCI repair, some works have exhibited that both mesenchymal stem cells (MSCs) and NSCs have the ability to enhance nerve regeneration and further improve SCI repair. Our results



show that exosomes derived from them promote the proliferation and migration of NSCs, respectively. We envision that they should be useful carriers of drugs and simultaneously act as modulators of NSCs, thus functioning as a valuable SCI treatment therapy. By virtue of the synthesized BSP to bind drug-carrying exosomes to collagen materials, the multifunctional scaffold showed superior performance for spinal cord repair by enhancing neural regeneration, reducing scar deposition, and promoting motor functional recovery in rats with complete SCI. This general strategy will open a way to fabricate diverse and multifunctional collagen scaffolds not only for SCI but also for the treatment of other diseases such as myocardial infarction, Alzheimer's disease, and tumors.

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## A fluorescent turn-on collagen hybridizing peptide probe for dynamic monitoring of collagen damage

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Title: A fluorescent turn-on collagen hybridizing peptide probe for dynamic monitoring of collagen damage

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Category: Enabling Technologies

Introduction: Collagen is the most abundant protein in mammals with a triple-helical structure, which can provide support and protection for cells and tissues. Numerous studies have shown that denatured collagen with disrupted structure existed in some diseases or injuries, such as tumors, fibrosis, and mechanical damage. In fact, denatured collagen is the marker for disease progression and also may have biological effects on the tissue microenvironment. Therefore, studying denatured collagen may provide new strategies for clinical diagnosis and treatment. Based on the characteristics of collagen structure and sequence, we previously developed a fluorescently-labeled Collagen Hybridizing Peptide (CHP) probe with repetitive Gly-Pro-Hyp triplets,[1] which can specifically recognize the unfolded collagen molecules present in disease and injury. Nevertheless, the information obtained from common fluorescence collagen hybridization imaging of tissues or in vivo was all static, while the kinetic information or dynamic monitoring of collagen damage and remodeling is almost impossible. Therefore, we recently developed a new class of CHP probes for dynamic monitoring of collagen damage and remodeling in tissues.

Subjects and Methods: Twisted Intramolecular Charge Transfer (TICT) is a non-radiative process of fluorescent molecules. Fluorophores with this property are not fluorescent in a polar environment or at the free rotation state.[2] Only when the molecule's rotation is restricted by the environment does it produce a strong fluorescent signal. Accordingly, we designed fluorescent turn-on CHP probes coupled with TICT molecules to investigate the kinetics of collagen hybridization and the dynamic process of collagen damage. We investigated the kinetics of denatured collagen hybridizing with CHP in gelatin and tissues, and validated the effect of wash-free staining with real-time and in situ imaging at the tissue and in vivo. Finally, we explored the dynamics of collagen structure disruption in tendon tissue under the action of collagenase and mechanical force.

Results: For kinetic studies and dynamic observations, we coupled TICT molecules on CHP to obtain the fluorescent turn-on collagen hybridizing peptide probe (TICT-CHP), which produced a fluorescent signal upon binding to denatured collagen. (1) In collagen hybridization kinetics studies, we found that CHP hybridized with denatured collagen quickly at the beginning, but took 2-3 h longer to reach saturation. (2) Using a turn-on CHP probe, we achieved wash-free staining and in situ, real-time imaging of denatured collagen in the scalded fin of zebrafish. (3) Finally, we performed a dynamic, in situ observation of the production of denatured collagen in tendon tissues under the action of collagenase or mechanical tensile loading. The results showed that collagenase destroyed tendon collagen very rapidly on a level of minutes. The mechanical damage of the collagen triple-helical structure started when tensile stretching ran into the yield strain range.

Discussion and Conclusion: By coupling TICT fluorescent molecules to CHP, we have successfully obtained the fluorescent turn-on collagen hybridizing peptide probes. Using the TICT-CHP, we found that hybridization between CHP and denatured collagen in tissues can occur immediately, and we could dynamically monitor the generation process of collagen damage in vivo and in vitro. In conclusion, the triple-helical fluorescent turn-on CHP probes, provide new tools to study the dynamics of collagens' structures and biology with the aim of offering new dynamic insights for collagen-related biomaterial and biomedical research.

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## Repair Of Osteochondral Defects With 3D Printed TGF- $\beta$ 1 Binding Peptide/GelMA Composite Biphasic Hydrogel Scaffolds

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**Introduction:** Articular cartilage and subchondral bone form a functional complex, and its structural and functional integrity is a prerequisite for ensuring normal joint movement and weight bearing. Maintaining the gradient characteristics of biochemical composition, structure, and biomechanics is the basis for the physical function of osteochondral complex. Due to the poor ability of selfrepair, osteochondral defect tends to progressively worsen, and eventually leading to joint dysfunction.

Reconstructing the normal structure and function of osteochondral tissue by constructing gradient-specific biomaterial scaffolds has been proven to be one of the most promising approaches. Gelatin Methacryloyl (GelMA) hydrogel materials have unique biological activity, physical and chemical properties and high potential for functionalization, are ideal materials for restoring osteochondral defect with gradient properties. In addition, 3D printing technology has unique advantages in finely controlling the structure, composition and mechanical properties of hydrogel scaffolds.

**Subjects and Methods:** TGF- $\beta$ 1 binding peptide (TBP) composite hydrogels with different TBP concentration were prepared. TBP200 group and TBPO group biphasic hydrogel scaffolds was prepared by 3D printing (TBP200 or TBPO as the cartilage layer and 10% GelMA/1% nHAp as the subchondral bone layer). Rat femoral trochlea osteochondral defect was established (N =3, n =16) was constructed and divided into TBP200 group, TBPO group and blank control group. At the 1st, 3rd and 6th week, gait analysis was performed to evaluate the knee function in each group.

Osteochondral specimens were obtained at the 3rd and 6th week. Micro-CT, HE staining, saffron O-fast green staining, the ICRS macroscopic score, the microscopic score systems for cartilage and subchondral bone repair were used to evaluate the effectiveness of 3D printed TBP composite biphasic hydrogel scaffolds.

**Results:** The gait analysis showed that the ratio of gait parameters (mean intensity, duty cycle, stride length, and stand phase) in each group gradually increased with the extension of postoperative time, and no significant difference was found between the parameters of the group TBP200 and TBPO. At the 3rd and 6th week, the ICRS macroscopic scores of the group TBP200 and TBPO were higher than those of the blank control group ( $p < 0.05$ ). However, no statistical difference between the group TBP200 and TBPO was found. The BMD and BV/TV in the defect area of the group TBP200 and TBPO were significantly higher than that of the blank control group at the 3rd and 6th week, but no significant difference between the group TBP200 and group TBPO. Mild synovitis was shown in each group at the 3rd week, and no synovitis appeared in the group TBP200 and TBPO at the 6th week. No obvious cartilage matrix staining in the defect area and the boundary with adjacent tissues was obvious in each group at the 3rd week. At the 6th week, the new tissues in the osteochondral defect of the group TBP200 and TBPO groups were well integrated with adjacent osteochondral tissues, and the cartilage-like matrix was formed to varying degrees on the surface of the new tissues. The microscopic scores for cartilage in the group TBP200 were significantly higher than those of the TBPO and blank group ( $p < 0.05$ ) at the 3rd and 6th week. The mean subchondral bone scores in the TBP200 group were higher than those in the TBPO group at the 3rd and 6th week, but no statistical differences existed.

**Discussion and Conclusion:** TGF- $\beta$ 1 binding peptide composite hydrogel scaffolds has good biocompatibility in animals, could safely and effectively promote the repair of osteochondral defects without relying on exogenous growth factors.

## Animal Study Of Patch Bridging Reconstruction Of Rotator Cuff Tears

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Category: Tissue Engineering and Regeneration

**Background:** Non-degradable synthetic materials have been used in clinical rotator cuff tear repairs in recent years due to their biostability and controllable mechanical properties, and have shown good functional outcomes with low retear rates. However, in vivo healing process and biomechanical properties after synthetic patch bridged rotator cuff repair has seldom been deeply studied.

**Introduction:** A knitted polyethylene terephthalate (PET) patch was fabricated for bridging reconstruction (PET group) in a rabbit full-thickness rotator cuff tear model (blank group), while the autologous tendon was used as a control (autograft group). The animals were euthanized and tissue samples were harvested for gross observation, histological and biomechanical analysis at 4, 8, 12 weeks postoperatively.

**Subjects and Methods:** No significant difference was found in the tendon-bone interface scores between the PET group and the autograft group by 4, 8, and 12 weeks. By 12 weeks, the formation of fibrocartilage and ingrowth of chondrocytes into the PET patch were observed in the PET group, while merely Sharpey fibers were observed in the autograft group. Besides, there was no significant difference in the tendon maturing score between the two groups by 4 and 8 weeks. By 12 weeks, the collagen fibers were more parallel in the PET group with collagen fibers ingrowth of the PET patch and distribution around PET fibers, and tendon maturing score was significantly higher than those in the autograft group ( $19.7 \pm 1.5$  vs.  $15.3 \pm 1.2$ ;  $p=0.011$ ). Furthermore, there was no significant difference in fatty infiltration between the two groups by 4 and 12 weeks, while fatty infiltration in the PET group by 8 weeks after surgery was significantly lower ( $5.5 \pm 0.6$  vs.  $10.2 \pm 1.9$ ;  $p=0.015$ ). Notably, fatty infiltration by 12 weeks in both PET group ( $10.1 \pm 3.5$  vs.  $3.0 \pm 2.9$ ;  $p=0.005$ ) and autograft group ( $14.1 \pm 3.7$  vs.  $5.6 \pm 3.5$ ;  $p=0.006$ ) was progressed compared with 4 weeks after surgery. As for biomechanical analysis, no significant differences were found in ultimate failure load, ultimate stress and stiffness between two groups by 4, 8, and 12 weeks. Ultimate failure load of the PET group by 8 weeks ( $125.3 \pm 13.6$  N,  $p>0.05$ ) and 12 weeks ( $139.5 \pm 18.3$  N,  $p>0.05$ ) was no different from native tendons ( $130.8 \pm 28.6$  N).

**Discussion and Conclusion:** The non-degradable synthetic PET patch could bridging reconstruct fullthickness rotator cuff tear in rabbit model and not only provide sustained mechanical support postoperatively, but also promote arrangement of regenerated collagen fiber and tendon maturation.

## Microfluidic Organ-on-a-Chips System to Investigate Outer Blood-Retina Barrier Using iPSC Derived RPE and Endothelial Cells

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**Introduction:** The retinal pigmented epithelium (RPE) along with the Bruch's membrane and the underlying choriocapillaris form the outer blood-retinal barrier (oBRB) that plays an important role in maintaining retinal function and homeostasis. Here, we combined human iPSC-derived RPE and endothelial cells (ECs) with organ-on-a-chip technology, to model the oBRB. Using this platform, we aim to investigate mechanisms of outer retinal degeneration.

**Methods:** Human iPSC were differentiated into RPE and ECs. Commercially available Emulate organ-chip microfluidic system was used as the basis to develop the oBRB model. The apical channel of the chip was seeded with immature iPSC-RPE cells and matured in the chip. ECs were deposited in the chip basal channel and allowed to form 3D monolayer around the channel to mimic the choriocapillaris. In order to characterize the functionality of the RPE-EC co-culture system in the chip, we assayed: (1) Dextran permeability, (2) fluid transport across apical to basal channels, and (3) RPE and EC morphology.

**Results:** Various genetically independent healthy and diseased iPSCs were differentiated into functional RPE and EC and cryopreserved at an immature stage. RPE cells seeded in the apical channel formed a monolayer of mature cells on the semi-permeable membrane and displayed highly pigmented polygonal morphology with ZO-1 expression. RPE tight junction integrity was confirmed using a fluorochrome conjugated dextran permeability assay. In addition, a fluid transport assay was performed to examine RPE transcytosis and homeostasis function. When compared to control chips seeded with only RPE cells, the fluid transport activity in RPE-EC chips was 2-fold higher and in range of physiological levels. These results support that both EC and RPE cells are critical components of fluid transport activity.

**Discussion and Conclusion:** The combination of a microfluidic organ-chip system with patient-specific iPSC derivatives has provided us with a non-invasive ex vivo model to study outer retinal physiology and a platform for drug discovery and toxicity screening.

**Acknowledgment:** This work was supported by NEI intramural IRP grant.

## A plasma-based, rapid, stable, and reagent-free method to create bioinstructive surfaces inside porous scaffolds

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### • Introduction

Rapidly developing tissue engineering (TE) approaches require three dimensional (3D) porous scaffolds with bio-functionality to promote tissue ingrowth and regulate cell behaviour. The bio-functionality of scaffolds is achieved by immobilising biomolecules onto the internal network of pores in the scaffolds. However, current bio-functionalization strategies rely on wet chemistry approaches, often limited by complicated and time-consuming processes and toxic residues.

### • Subjects and Methods

Radicals embedded in polymeric surfaces via energetic ion bombardment through plasma immersion ion implantation (PIII) technology enable covalent immobilisation of biomolecules through a low cost, versatile, and environmentally friendly process without the above issues. However, current plasma-based technologies have not yet successfully achieved uniform surface treatment inside porous TE scaffolds. Such approaches rely on the diffusion of reactive species into the pores with limited penetration depths (4 mm). Here, we report the development of a new PIII-based approach to homogeneously surface engineer 3D porous scaffolds (30 mm long) using a specifically designed plasma reactor, followed by validation experiments in the context of mesenchymal stem cell (MSC) expansion.

### • Results

The new approach applies a high voltage electrode surrounding the polymeric scaffolds for their homogenous PBPIII activation. Voltages (4- 8 kV) and working pressures (1-3 Torr) were tuned to optimise the PIII activation inside high impact polystyrene scaffolds with pore sizes of 250  $\mu\text{m}$ . Using micro-Fourier-transform infrared spectroscopy, the homogeneous surface modification of the PBPIII-treated scaffolds was confirmed by mapping the level of surface activation evidenced by the creation of O-H groups. Fibroblast growth factor 2 was covalently immobilised to the surfaces of the scaffolds, promoting both MSC adhesion and proliferation.

### • Conclusion

These promising results show that the porous network of the scaffolds can be homogeneously PBPIII-treated using this new strategy, providing excellent bio-functional platforms for MSC expansion and other applications in tissue engineering and regenerative medicine. Further development of this method in different geometries and materials will also be reported.

## Construction of liver sinusoid like tissue using hiPSC-derived liver sinusoidal endothelial cells

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• Introduction: After digested foods or drugs are absorbed from the intestine, they are transported via the portal vein to the hepatic sinusoids. Liver sinusoidal endothelial cells (LSECs), which comprise the liver sinusoids, are the first cell types to encounter many xenobiotics. They express a wide variety of scavenger receptors responding to various xenobiotics, and their ability to endocytose leads to the elimination of xenobiotics and immune tolerance to suppress autoimmune diseases. As so, LSECs are important cell to discuss about the liver sinusoids. In a previous study, we developed a liver tissue chip cultured in a microfluidic device. It revealed the cell polarity constituting the liver tissue and the flow rate of the culture medium are important to maintain a function like liver tissue. Therefore, we believe that in vitro liver tissue engineering using LSECs, and a microfluidic device that mimics cell polarity will provide a liver tissue model that will significantly improve preclinical studies.

• Subjects and Methods: The human iPS cell line 253G (provided by RIKEN) was maintained in feeder-free culture, and differentiation of 253G into hepatocyte lineage cells (Hepatocytes) and LSECs was attempted by adding Activin A, BMP-4, HGF, VEGF, 8-Br-cAMP, SB-431542, etc. under the appropriate conditions. In some experiments, HepG2 with DsRed induced cells, RH4 cells, were used. A membrane-anchored microfluidic device which can circulate two types of culture media was designed to construct liver sinusoidal tissue model.

• Results: In endothelial cells differentiated from human iPS cells, the LSEC-specific gene expression as well as the function were confirmed, which were not observed in human umbilical vein endothelial cells (HUVECs). Hepatocytes differentiated from human iPS cells have shown the increase in hepatocyte-specific genes. Using cell culture inserts, LSEChiPSC and HepatocytehiPSC were co-cultured in two layers, the specific gene expression of both cells was maintained for several days. To reveal the importance of microenvironment, microfluidic device was used to mimic the liver sinusoidal tissue.

• Discussion and Conclusion: In the liver sinusoid tissue model constructed in this study, the expression of each specific gene was enhanced and maintained compared to that in monoculture. This is thought to be due to the maintenance of cell polarity by co-culture, which led to the maintenance of each gene expression. This is expected to be a sign that functional long-term culture and construction of a liver sinusoidal tissue model derived from human cells will become possible, which have been difficult to achieve in the past. We believe that the application of this technology to microfluidic devices will lead to the development of liver tissue chips that can be used in liver research. This liver sinusoidal tissue model is expected to be applied to drug metabolism research and antiviral drug screening.

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## Three-Dimensional Printed Double-layer Artificial Skin With Antibacterial, Angiogenic Potential And Anti-scar For Skin Regeneration And Wound Healing

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**Introduction:** Post-burn infection and scar contracture are two major challenges in the treatment of burns. These can significantly impact patients' physical and mental health, highlighting the pressing need to find solutions for full-layer burn wound repair. Nevertheless, there are limited materials that can be used for fighting infection, promoting wound healing and combatting scar contracture. Therefore, it is urgent to construct artificial skin for skin regeneration with antibacterial properties and the function of suppressing scar contracture. This study aims to investigate the use of 3D printing technology to precisely prepare two types of double-layer artificial skin, one for post-burn infected wounds to fight infection and promote wound healing, and the other for promoting efficient wound healing while inhibiting scar contracture.

**Methods:** In this study, we prepared Methacrylated silk fibroin (SFMA), gelatin methacryloyl (GelMA), methacrylated hyaluronic acid (HAMA), copper-epigallocatechin gallate (Cu-EGCG), and human immortalized keratinocytes (HaCaT) cells to be used as double-layer skin materials for projection light-curing 3D precision printing. Specifically, SFMA/GelMA/(HaCaT cells) were used as the upper layer while GelMA/HAMA/Cu-EGCG were used as the lower layer. To investigate the synthesis and skin-related properties of the materials, a series of materials science experiments were conducted including scanning electron microscopy, x-ray spectral analysis, nuclear magnetic resonance spectrometry, compression properties, degradation, and copper ion release experiments, among others. Furthermore, cellular experiments were performed to investigate the survival and properties of cells in in vitro materials such as cell proliferation experiments, live/dead staining, tube formation assays, tracing of live cells, cytoskeleton staining, etc. The anti-infection properties were explored through in vitro and in vivo antibacterial assays. Animal experiments were conducted by constructing two types of full-thickness post-scald models using SD rats. The first is an infection model using *E. coli* and *S. aureus* bacteria for infection after cutting scab, and the second is a non-infection model with an upper layer containing HaCaT cells. After 3, 7, 10, and 14 days, the double-layer skin's role in efficiently promoting wound healing was investigated using HE, Masson staining, Sirius scarlet staining, immunofluorescence staining, PCR, and Western blot experiment.

**Results:**

1. Material science experiments proved that the materials met the requirements as skin mechanics, with suitable swelling, degradation rate and copper ion release properties.
2. Cellular experiments indicated that both upper and lower layer materials had good biocompatibility, the upper layer, SFMA/GelMA, has an obvious effect of promoting cell proliferation. In the lower layer, the ability to promote angiogenic was stronger as the concentration of Cu-EGCG increased. And the cell survived and proliferate well in the 3D printed double-layer material.
3. Animal experiments demonstrated that the double-layer skin material group was closer to the normal group and had the best healing quality. The infected animal models demonstrated the anti-inflammatory, anti-infective and pro-angiogenic properties of the material, and the animal model using the material with HaCaT cells healed best with its anti-scarring effect.

**Discussion and conclusion:** The 3D printed double-layer artificial skin prepared in this study has good mechanical properties and biocompatibility, significant anti-inflammatory, antibacterial and angiogenic properties, while co-cultured with HaCaT cells possessing anti-scarring and contracture potential, thus holding great clinical promise for full-thickness burn wound regeneration.

## Plasma-engineered Solid-hydrogel Hybrid Structures for the Fabrication of Nerve Guide Conduits

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Category: Tissue Engineering and Regeneration

**Introduction:** Artificial nerve guide conduits (NGCs) are promising as a treatment solution of peripheral nerve injuries (PNIs) to overcome the limitation of the current gold standard, such as donor site morbidity, the inadequate available donor nerve tissues for autologous transplantation, and the potential risk of neuroma [1]. NGCs combines the potential of cell therapies and pharmacological interventions and have been approved for clinical use. However, the performance of current NGCs technologies is variable and not consistently on par with or superior to autografts. Plasma immersion ion implantation (PIII) is an emerging, dry, and green technique that enables covalent immobilisation of biomolecules and/or hydrogels onto polymers [2] for establishing a novel NGC. Here we report the development of a hybrid solid-hydrogel NGC surface using this novel PIII approach. PIII was used to embed highly reactive radicals inside polytetrafluoroethylene (PTFE) surfaces with followed by covalent attachment of a wide range of biomolecules and hydrogels without using additional chemical reagents, cross-linkers, or initiators. The developed hybrid solid-hydrogel structures can be used to synthesize versatile composite NGCs for improving peripheral nerve regeneration and rehabilitation of neurological function.

**Subjects and Methods:** Our PIII approach utilizes high voltage pulses to implant ions from plasma into the polymeric surface, embedding a high concentration of reactive radicals to a significant depth (~ 100 nm). 2D PTFE has been selected as a model solid substrate. The number of free radicals within the samples' surface and surface chemistry were demonstrated by electron paramagnetic resonance spectroscopy (EPR) and X-ray photoelectron spectroscopy (XPS) respectively. Surface characterization was conducted by attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR). Surface hydrophobicity and hydrophilicity change were tested by a water tensiometer. Different hydrogels, including GelMA, acrylamide, chitosan, and alginate, were attached to both untreated and PIII-treated PTFE. Their covalent binding between untreated/PIII-treated surface and hydrogels was confirmed by ATR-FTIR and XPS. Stability and strength of attachment were detected through the water reswelling test and T-peel off tape test. Surface and cross-section topography of PTFE and hydrogels was observed using scanning electron microscopy (SEM).

**Results and Discussion:** Results of EPR showed the generation of enormous surface-embedded radicals in the PIII-treated PTFE. XPS provided insights on the changes of surface chemistry as a result of PIII. The wettability of PIII-treated surfaces has been improved, as confirmed by decreased surface

water contact angles. In addition, ATR-FTIR demonstrated various types of hydrogels were subsequently strongly attached to the PIII-treated PTFE. Reswelling testing results proved a stable and strong bonding between the hydrogel and PIII-treated surfaces in an aqueous environment. The mechanical adhesion tests demonstrated that the adhesion strength of hydrogels attached to PIII-treated surfaces has been significantly enhanced compared with the untreated surface. The insights obtained from these fundamental 2D studies will be applied to create 3D composite nerve scaffolds.

Conclusion: These highly promising results prove that PIII has enabled strong binding between the polymeric solid surfaces and various types of hydrogels. This approach can be used to synthesize versatile composite NGCs for improving peripheral nerve regeneration and rehabilitation of neurological function.

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## A self-growing osteoinductive polymeric framework facilitates endogenous osteogenesis by continuously capturing calcium ions

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Title: A self-growing osteoinductive polymeric framework facilitates endogenous osteogenesis by continuously capturing calcium ions

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Category: Tissue engineering and Regeneration

Background: Bone regeneration is a complex yet well-organized process of continuous remodeling that involves several sequential steps such as endogenous cell homing, angiogenesis, osteogenesis, and biomineral deposition.[1,2] Typical bone grafts lack the complexity of biological tissues and are usually considered parts of static, closed systems that do not exchange substances with their surroundings. Therefore, it remains both challenging and important to obtain self-growing osteoinductive scaffolds that can continuously bind bioactive factors, exchange matrix with the surrounding microenvironment, and orchestrate bone regeneration by regulating stem cells and the local milieu in vivo.

Methods: In this study, a self-growing osteoinductive scaffold (SOPF) was developed using dopamine-interlaced polymeric framework that could continuously capture calcium ions by exchanging matrix with the surrounding microenvironment and we tested its effects on bone formation in vitro and in vivo.

Results: Due to the optimal, stable, interconnected and porous structure, SOPF stimulated effective osseointegration with host bone tissue by sustainably initiating interfacial Ca<sup>2+</sup> enrichment and biomineralization in vivo. Additionally, the SOPF mediated the M2 phenotypic polarization of macrophages and recruited endogenous stem cells to achieve vascularized osteogenesis.

Transcriptomic analysis further revealed that the mechanism of osteoinduction involved the activation of the focal adhesion/extracellular matrix- receptor interaction/PI3K-Akt signaling cascade, which was further verified in rabbit ( $\Phi = 10$  mm; new bone cover ratio: 84%) and beagle dog ( $\Phi = 15$  mm; new bone cover ratio: 38%) cranial defect models.

Scheme shows the SOPF obtained by dopamine interlaced polymeric framework facilitates endogenous osteogenesis by continuously capturing calcium ions.

Discussion and Conclusion: We demonstrated that the SOPF can continuously capture calcium ions and exchange matrix with the surrounding microenvironment. The approach enabled the widespread deposition of apatite crystals with ultrastructural organization and elemental composition comparable to that of human bone, and promoted the osteogenic and angiogenic differentiation of BMSCs. Due to its optimal stable spatial structure and components, the SOPF achieved effective osseointegration with host bone tissue by sustainably initiating interfacial Ca<sup>2+</sup> enrichment and biomineralization in vivo. Moreover, the SOPF mediated M2 macrophage polarization and recruited ESCs to achieve vascularized osteogenesis. The overall results highlighted that this model may have important implications for ongoing efforts to generate novel osteoinductive polymeric biomaterials and better understand bone physiology.

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## Engineered Endothelium Model Enables Recapitulation of Vascular Function and Foam Cell Development

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Category: Design and Application of Biomaterials

Background: Endothelium basement membrane (BM) interfaces endothelial cells (ECs) and pericyte. Here, we developed an artificial BM (aBM) that consists of human fibroblast-derived matrix (hFDM) embedded in the porous-polyvinyl alcohol (p-PVA) hydrogel. We hypothesize that our fabricated aBM enables direct interactions of ECs and pericytes where it ultimately gives a physiological as well as functional relevance of the endothelium. To this end, by using this aBM, we prepared an engineered endothelium model (EEM) and developed an early stage of atherosclerosis model via foam cell formation.

Methods: Human lung fibroblast cells (WI-38, ATCC) were harnessed to obtain decellularized ECM according to our previous protocol. PVA solution was prepared by dissolving PVA (Sigma Aldrich) into the DMSO and DW. The solution was then stirred at 90°C for 2 hr and then added with NaCl (Junsei Chemical) and SiO<sub>2</sub> (Sigma-Aldrich). Such mixed PVA solution was then poured on the human fibroblast-derived matrix (hFDM) and covered with another hFDM on glass substrate. The sample was then freeze-thawed and dialyzed with DW to obtain p-PVA membrane. Cytotoxicity of p-PVA was evaluated by seeding HUVECs (Lonza) in EBM-2 supplemented with EGM-2 Kits (Lonza). Permeability assay is conducted using FITC-labelled dextran with two different types of molecular weight, 10 kDa and 70kDa (Sigma Aldrich). Additionally, nitric oxide (NO) secretion of EEM was measured according to Griess assay protocol using 1X Griess reagent (Sigma Aldrich) solution. The images from the immunofluorescence staining are acquired using confocal microscopy (Zeiss, Germany). Furthermore, we also utilized time lapse microscope (Zeiss) to track the migration velocity of the THP-1 cells both before and after the trans-endothelial migration. We also use both western blot technique and real time PCR for protein quantification and gene expression level quantification, respectively.

Results: Blood vessels play a critical role in the maintenance of human physiological function. Here, we propose an engineered endothelium model (EEM) for vascular study and disease model development. Our EEM contains an artificial basement membrane (aBM), where porous polyvinyl alcohol hydrogel was securely integrated with human fibroblast derived extracellular matrix (hFDM) on both sides, in which human endothelial cells (ECs) and pericytes were settled down, respectively. Our EEM successfully developed cell-cell adherens junction of vascular endothelial cadherin (VE-cad) and also showed a solid barrier function as assessed via permeability test using FITC-dextran. An important endothelium function was also confirmed via nitric oxide (NO) secretion with the progress of the time. We found that NO secretion level was mediated by Hb- $\alpha$ 1. Moreover, we also found evidence of interaction of ECs and pericytes via Hb- $\alpha$ 1 where it was detected between the layer of ECs and pericytes. The EEM under inflammatory milieu with tumor necrosis factor (TNF)- $\alpha$  treatment

exhibited disrupted VE-cad structure initially but fully recovered with time. Interestingly, our EEM enabled transendothelial migration of human monocytes (THP-1) and following foam cells formation, an early precursor of atherosclerotic development as treated with Ox-LDL and MCP-1.

**Discussion and Conclusion:** We have developed an aBM that composed of p-PVA embedded hFDM and co-cultured with ECs and pericytes which we termed as EEM. In normal condition endothelial cells in our system formed a robust VE-cad that mediated lower permeability this is then led to the higher secretion of NO. In inflammatory condition using TNF- $\alpha$ , our EEM showed dynamic response which then subsequently allow the TEM of the THP-1 to reside under endothelial cells and differentiated into macrophage. These macrophages were then able to form the initial stage of the atherosclerosis with the accumulation of the lipid once treated with the Ox-LDL. In conclusion, our EEM demonstrated its feasibility as an in vitro vascular model, where it can recapitulate endothelial function and dysfunction and the ability to form foam cells as the marker of the initial atherosclerosis model.

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## Supramolecular Nitric Oxide Depot for Hypoxic Tumor Vessel Normalization and Radiosensitization

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Title: Supramolecular Nitric Oxide Depot for Hypoxic Tumor Vessel Normalization and Radiosensitization

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Category: Design and Application of Biomaterials

**Background:** As an important gas molecule with multiple biological functions related to blood vessels nitric oxide (NO) has several effects on hypoxic tumor. Since direct use of NO gas is problematic in vivo, many exogenous NO donors have been developed as sensitizers to enhance radiation therapy. Currently, most of the NO delivery systems focused on large amount of release of NO in a relatively short time period, which were not suitable for vessel normalization. A delivery material that could release NO sustainably with precise control in both quantity and duration is still lacking.

**Methods:** In this study, we designed a supramolecular hydrogel as a NO depot for continuous delivery of NO on-demand. First, after the hydrogel being injected within the tumor as an NO depot,  $\beta$ -Gal in the tumor environment will trigger the release of NO continuously and offer the locally sustained delivery of NO at low dosage. Second, large amount of NO could be released when plenty of  $\beta$ -Gal was intravenous injected immediately before irradiation.

**Results:** The experimental results showed that the overexpression of  $\beta$ -Gal could trigger the controllable and sustained release of NO from supraNO hydrogel. The NO released by the catalysis of  $\beta$ -Gal from HUVECs had no harm to them. However, NO released by  $\beta$ -Gal catalysis of B16 exhibited obvious toxicity after SupraNO treatment under hypoxic conditions, the irradiating damage to DNA can be restored to the normoxic level, and the degree of recovery depends on the amount of  $\beta$ -Gal. SupraNO depot has great radiosensitization potential by combining low-dose long-time release of NO with high-dose short-term release of NO, which could significantly improve the tumor suppressive efficiency of IR. The significant improvement of vessel normalization in LH-SupraNO+IR group could be attributed to the combination effect of sustainable released low-dose NO and the elimination of tumor cells by high-dose NO and IR, by which the cytokines secreted by tumor cells that would induce vessel abnormalities were reduced massively.

**Discussion and Conclusion:** We have rationally designed a NO releasing depot named SupraNO, which is composed of two parts: the self-assembling peptide and the caged-NO donor. The SupraNO provides an effective solution for both vessel normalization by low-dose long-time NO supply and radiosensitization by temporary high-dose stimulation in hypoxic tumor. The design of SupraNO could be further improved by replacing the self-assembling peptide with its D-enantiomer, which could prolong the retention time in the body and reduce the frequency of SupraNO administration by delaying the protease degradation.

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## Spatial and temporal single cell multi-omic atlas of endometrial injury and regeneration

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Title: Spatial and temporal single cell multi-omic atlas of endometrial injury and regeneration

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Category: Tissue Engineering and Regeneration

**Background:** The full-thickness endometrium is essential for fertilization and embryonic development, with exhibit remarkable plasticity and repeated injury and regeneration. The highly dynamic properties of repeated injury and scar-less repair during the menstrual cycle make it an ideal model to study tissue regeneration. Our understanding of the endometrial regeneration after injury is limited by an incomplete molecular characterization of the cell populations responsible for the organ functions.

**Methods:** In this study, we used mouse full thickness endometrial injury model, and conducted time series (12h, 1d, 2d, 4d, 7d, 14d, 28d, 56d) bulk RNA-seq, scRNA-seq and scATAC-seq after injury to characterize the spatial and temporal single cell multi-omic atlas of endometrial injury and regeneration.

**Results:** We dissected the temporal specific cell subpopulations as well as their interactions with their niche, and also revealed sequential gene regulatory program of endometrial injury and regeneration. With the acute inflammation occurred shortly after injury (12h), and re-vascularization persisted from 2-4 days post injury (dpi). And the endometrial regeneration peaked at 4 dpi, and reepithelization completed at 14-28 dpi. We also characterized regenerative signaling molecules (IGF1, PDGF etc) regulating endometrial regeneration.

**Discussion and conclusion:** The spatial and temporal single cell multi-omic atlas of endometrial injury and regeneration revealed the cell subpopulations, their communications and the sequential gene regulatory programs during the endometrial injury and regeneration, which provide insight into the biology of tissue regeneration and the development of regenerative medicine treatments against endometrial damage and intrauterine adhesion.

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## Biomaterial Topography Regulates Stem Cell Fate through Controlling Force Mediated Chromatin Accessibility

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**Introduction:** Topography is one of the important physical cues that biomaterials regulate stem cell fate. Previous studies shown that aligned topography materials promote stem cell differentiation into tendon, muscle and nerve lineage, while random topography materials promote stem cell differentiation into bone and cartilage lineage. These results mean that certain topographic structure induces several directions of cell differentiation, but how topographic cues guide “multiple cell fate decision” is largely unknown. Traditional mechanism research strategies, such as changes in the level of mechanical signals and epigenetic markers, cannot precisely explain the “multiple cell fate decision” property of topography, new research perspectives and strategies are needed.

**Subjects and methods:** In this study, we took aligned and random fiber scaffolds as research model to study the morphology and cell fate of adipose derived stem cells (ADSC) cultured on them. We intended to use transposase accessible chromatin sequencing as the main method to explore the stem cell nuclear stress and chromatin opening/closing pattern, as well as to identify topographic responsive chromatin regions and their key regulatory factors.

**Results:** Firstly, we verified the “multiple cell fate decision” property of topographic cues. We found that stem cells exhibited different mechanical conduction patterns and nuclear morphology in aligned and random topographies. ATAC-seq analysis showed that aligned topography induces specific open chromatin regions around myogenic and neurogenic genes while specifically closes chromatin regions around osteogenic and chondrogenic genes which were specifically opened by random topography. Besides, motif analysis showed that the specific open chromatin regions of ADSC showed enrichment of binding site of nuclear factor NF1 in aligned topography but showed enrichment of binding site of PITX1 in the random topography.

**Discussion and Conclusion:** Taken together, our work uncovers that topographic cues shape the nucleus structure through force conduction, then remodel the chromatin accessibility into a topographic specific profile and finally guide the specific “multiple cell fate decision” of stem cells. These findings provide new insights into the regulation of stem cell multipotency, as well as a resource and model to research the interaction between cells and materials.

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## Study of Paraquat-induced Pulmonary Fibrosis Using Biomimetic Micro-lung Chips

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Category: Tissue Engineering and Regeneration

Background: Paraquat causes severe pulmonary fibrosis or even death. The pathogenesis of pulmonary fibrosis is complex, and the pathomechanism of pulmonary fibrosis remains unclear. Therefore, developing a valuable preclinical model to investigate the pathogenesis of lung diseases and identify effective treatments for pulmonary fibrosis is imperative. Organ-on-a-chip systems have attracted substantial interest in recent years owing to their potential for drug discovery and elucidating disease pathophysiology.

Methods: In this work, we designed a biomimetic multichannel micro-lung chip to imitate the organisational interface between the alveolar epithelium and the interstitial space in the lung for studying paraquat-induced pulmonary fibrosis.

Results: Co-culture of A549 and MRC-5 cells in the micro-lung chip promotes paraquat-induced pulmonary fibrosis. A model using contactless microchannels was designed to compare with the co-culture model. The A549 and MRC-5 cells co-cultured with paraquat in the micro-lung chip had the higher cell viability than cells monocultured in chips. Moreover, Co-culturing lung epithelial cells and fibroblasts exhibited more severe epithelial-mesenchymal transition after treatment with paraquat. These results confirmed that interactions between A549 and MRC-5 cells in micro-lung chips protected cells against destruction from paraquat.

Discussion and Conclusion: Our results show that the micro-lung chip reproduces pulmonary fibrosis caused by paraquat in vitro, thereby providing a good pathological model to investigate pulmonary fibrosis and screen potential drugs.

## Denatured Collagen-Targeted PET Radiotracer for Early Detection of Pancreatic Cancer

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**Background:** Pancreatic ductal adenocarcinoma (PDAC) is a deadly malignancy with a dire prognosis due to its aggressive biology and lack of effective tools for diagnosis at an early stage. The development of highly specific noninvasive imaging probes for PDAC is essential to improve diagnostic accuracy and even to achieve early detection. A dense fibrous extracellular matrix (ECM) is present in PDAC and undergoes stromal remodeling during tumor progression. Therefore, targeting the remodeled matrix in PDAC offers new possibilities for diagnosing PDAC.

**Methods:** In this work, we designed and constructed a <sup>68</sup>Ga labeled PET probe (<sup>68</sup>Ga-CHP) for specific targeting to degraded and denatured collagen molecules in the remodeled ECM based on the collagen hybridizing peptide (CHP) previously developed by us. The ability of <sup>68</sup>Ga-CHP to detect PDAC and the biodistribution of the probe were evaluated using PET/CT imaging in an orthotopic mouse model (CFPAC-1). Further, the probe was validated in KPC mice, a genetically engineered mouse model, that could spontaneously form pancreatic intraepithelial neoplasia (PanIN) and PDAC, to display the potential of <sup>68</sup>Ga-CHP in the detection of early PDAC lesions and malignancies.

**Results:** Pathological staining showed that there was substantial collagen remodeling in human pancreatic cancer tissue samples, CHP could indicate tumor foci and target the denatured collagen in PDAC. In the orthotopic mouse model, <sup>68</sup>Ga-CHP exhibited significant tumor accumulation, in contrast to the non-targeting control peptide probe. Moreover, the <sup>68</sup>Ga-CHP uptake in tumors was significantly blocked by an excess of unlabeled CHP, further confirming the targeting properties of the probe. The isolated biodistribution and immunohistochemical results were consistent with the in vivo imaging results. In the genetically engineered mouse model, which better matches human pathology, <sup>68</sup>Ga-CHP was able to detect not only PDAC tumors but even early PanIN lesions, implying a potential for early diagnosis of pancreatic cancer.

**Discussion and Conclusion:** We demonstrated that the denatured collagen is a specific and sensitive biomarker in the development of PDAC even in precancerous lesions. The denatured collagen-targeting <sup>68</sup>Ga-CHP probe can accurately detect precancerous lesions and malignant tumors in PDAC and has great potential for clinical translation.

## Multifrequency control of Faraday wave bioassembly for constructing multiscale hPSC-derived neuronal networks

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**Introduction:** Bioassembly is recently regarded as a critical alternative biofabrication technical route to bioprinting by the International Society of Biofabrication. Since it can directly manipulate millions of live cells to form multicellular structures with close intercellular proximity, it improves contact-dependent cell communication and promotes the emergence of tissue-specific functions. Particularly, a variety of acoustic bioassembly techniques based on Faraday waves, bulk acoustic waves, and surface acoustic waves have been increasingly reported in biofabrication due to their advantages of high tunability, biocompatibility, and efficiency[1-3]. Acoustic bioassembly techniques are currently limited to generating cytoarchitecture with a single characteristic length that is determined by half acoustic wavelength. However, cytoarchitectures in natural tissues are usually highly complex and contain multiscale cellular structures, which represents a great challenge for acoustic bioassembly.

**Subjects and Methods:** We developed a multifrequency-driven Faraday wave bioassembly technique for constructing multiscale cellular structures. Specifically, we investigated the effects of acoustic frequency, phase, and amplitude on multifrequency-driven Faraday wave bioassembly by numerical simulations and microparticle assembly experiments. Furthermore, we utilized two-frequency ( $f_1=55$  Hz,  $f_2=145$  Hz) driven Faraday wave bioassembly to construct multiscale neuronal networks. hiPSC-derived neural stem cells were assembled in Matrigel, followed by 21-day neuronal differentiation. Further, bright-field imaging, immunofluorescence staining, and neuroelectrophysiological analysis were performed to characterize brain-specific neural types, structures, and functions.

**Results:** Bright-field imaging indicates the formation of multiscale neuronal networks. Immunofluorescence staining demonstrated neurons and astrocytes in the assembled constructs with multiscale synaptic connectivity. Neuroelectrophysiological analysis further demonstrated improved electrical activity in terms of the number of spikes, bursts, and mean firing rate compared to neuronal networks developed by single-frequency driven assembly and random groups.

**Discussion and Conclusion:** We reported a novel acoustic bioassembly technique that employed multifrequency control of Faraday waves to form multiscale cellular structures. This technique overcomes the structural limit of single-frequency driven acoustic bioassembly and potentially emulates complex cytoarchitecture in natural tissues. Additionally, this technique preserves the traditional advantages of bioassembly and enables to generate multiscale structures in a few seconds. Using this technique, we constructed functional neuronal networks with multiscale connectivity that displayed higher electrical activities than the one developed by one-frequency driven bioassembly. We expect this technique will find wide applications in tissue engineering and regenerative medicine.

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## Evaluating the Impact of Air Pollution on Voice and Upper Airway Health using an iPSC-derived Vocal Fold Mucosa Organ-on-a-Chip

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Title: Evaluating the Impact of Air Pollution on Voice and Upper Airway Health using an iPSC-derived Vocal Fold Mucosa Organ-on-a-Chip

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Category: Tissue Engineering and Regeneration

Background: Coarse particulate matter with an aerodynamic diameter of 10 µm (PM10) is a major air pollutant speculated to impact voice and upper airway (VUA) health. Currently, the association between PM10 and the VUA health remain experimentally undefined.

One critical challenge is the lack of in vitro models that can fully recapitulate the complex structure and physiology of the vocal folds within the upper airway tract. For instance, conventional transwell models cannot replicate the physiological biomechanical cues (e.g. shear fluid flow) that cells of the vocal fold mucosa, such as epithelial cells and fibroblasts, would experience in vivo.

Another challenge of developing vocal fold mucosa models is the limited lifespan of primary vocal fold epithelial cells in vitro. iPSC culturing technology has been utilized to derive vocal fold epithelial cells that, in a transwell set-up, were co-cultured with vocal fold fibroblasts. The resultant mucosal construct retained structural and functional features of the native vocal folds.<sup>1</sup>

However, transwell models provide cells with fluid volumes that can exceed 1000 times what cells experience within native tissue. This can create challenges for exposing cells to soluble drugs or toxins in physiologically relevant conditions. Microfluidic culture technology has been effectively applied to replicate organ-level responses, with the microscale nature of such systems providing cells with fluid volumes relative to their native environment.

As such, this study aimed to microengineer an iPSC-derived vocal fold mucosa organ-on-a-chip (VF-OOAC) model to study the impact of pollutant exposure on vocal fold biology.

Methods: A microfluidic device (BE-Transflow, BEOnChip) consisting of a microfluidic channel and culture well separated by a porous polycarbonate membrane (10 µm thick, 0.4 µm pore size) was used for cell culture in this study. iPSC-derived vocal fold epithelial cells and primary vocal fold fibroblasts (pVFFs) were co-cultured in microfluidic devices under perfused conditions for 3 weeks to form the base VF-OOAC model (Figure 1). The well of the microfluidic device contained a 3D collagen gel with embedded pVFFs to support the proliferation and differentiation of iPSC-derived epithelial cells seeded atop the gel. A monolayer of pVFFs was cultured in the microfluidic channel to represent interstitial tissue flow and facilitate fluid shear cell signalling pathways. Media was perfused

continuously through the channel of the chip at a rate of 40  $\mu\text{L}/\text{h}$ . Transwell 3D cultures were used as controls.

Following vocal fold mucosa development, 100  $\mu\text{g}/\text{mL}$  PM10 was applied to the culture well for 24-hr. Cultures continued to be perfused at a rate of 40  $\mu\text{L}/\text{h}$  throughout PM10 exposure. Microfluidic cultures without PM10 were used as negative dose controls and transwells (with and without PM10) as experimental controls.

Structural and functional markers of the human vocal fold mucosa were assessed using immunocytochemistry (K13, Vimentin, La- $\alpha$ -5) and qPCR (K5, K13, p63, ZO-1, GJA1, MUC1, HAS2, HAS3, PIEZO1). Additional markers (IL1 $\alpha$ , IL1 $\beta$ , IL6, IL8, TNF $\alpha$ ) were used to evaluate the inflammatory response of mucosae to PM10 exposure.

Results: Compared to transwell controls, significantly higher gene expression related to tight junctions (ZO-1), gap junctions (GJA1), mucus production (MUC1), and mechanosensitivity (PIEZO1) were observed in the microfluidic cultures. Microscopy images confirmed the presence of a multi-layered epithelium and basement membrane in addition to elongated fibroblast morphology within the collagen gel. Compared to negative controls, PM10 exposure in microfluidic induced increased keratin production (K13) but had no significant effect on all other genes. Transwell cultures demonstrated no significant changes in any genes in response to PM10 exposure.

#### Discussion & Conclusion:

Our results confirmed the presence of a differentiated, mature epithelium in VF-OOAC cultures, as indicated by a strong gene expression associated with epithelial barrier integrity, cell communication, and mucus production.

Compared to conventional transwell models, perfusion of the VF-OOAC provided cells with physiologically relevant biomechanical cues and prevented the build-up of waste and acidity in the culture. Our microscale culture provided a cell-to-media ratio that ensured cell-cell interactions and signalling more closely aligned with in vivo behaviour.

The upregulation of cytokeratin marker K13 may be an early marker associated with PM10-induced hyperplasia, i.e., excessive cellular proliferation. However, hyperplasia is a chronic condition from prolonged exposure to air pollutants. Further research that utilizes perfusion-based culture to maintain cell viability and proliferation will enable the exploration of longer-term exposure to PM10 to evaluate its impact on vocal health.

Our work showed, for the very first time, the assembly of an iPSC-derived vocal fold mucosa based on microfluidic technology. Future work will include connecting this model to other airway organ-on-a-chip models (e.g. trachea, nasal) to explore the interactions that occur in the multi-organ innate immune response of the airway to air pollutants.

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## Near-field acoustic bioassembly of human cortical microtissues from hiPSC-derived neural progenitors and neurons

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Title: Near-field acoustic bioassembly of human cortical microtissues from hiPSC-derived neural progenitors and neurons

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Category: Enabling Technologies

Background: Development of human biologically relevant and clinically relevant cerebral cortex models is demanded by mechanistic studies of human cerebral cortex-associated neurological diseases and discovery of preclinical neurological drug candidates. However, there are very few humanized neural models available for reconstituting structures and functions of matured cortical tissues and faithfully recapitulating neurological diseases[1-2].

Methods: In this work, we demonstrated rational design of human-sourced brain-like cortical tissues by reverse engineering and bio-inspired design. To implement this design, we employed an near-field acoustic waves to assemble hiPSC-derived neural progenitors and neurons separately into concentric tissue layers in a label-free and contact-free manner, followed by subsequent neural differentiation and culture. Immunofluorescence staining, and microelectrode array analysis were used to characterize the cortical microtissues. Infection models were generated by incubation with the titer of HSV-1(100,000 pfu/cortical microtissue) in the culture medium for one day.

Results: The generated cortical microtissues encapsulated neuronal microanatomy of human cerebral-cortex tissue with six-layered neuronal architecture, 400-micrometer interlayer distance, synaptic connections between interlayers, and neuroelectrophysiological transmission. Furthermore, the cortical microtissues were successfully infected with HSV-1 virus and displayed the HSV-induced pathogenesis associated with Alzheimer's disease, including neuron loss and the expression of A $\beta$ .

Discussion and conclusion: We developed a bioassembly technique enabling the generation of a six-layer concentric tissue architecture using near-field acoustic waves. Based on this acoustic bioassembly technique, we further developed two strategies for forming cortical microtissues. The acoustically assembled cortical microtissues encapsulated the hallmark features of human cerebral-cortex tissue, including interlayer distance, synaptic connectivity, and electrical activities. Compared to cerebral organoids that are generated via neurodevelopmental strategy, our bioengineered cortical microtissues faithfully emulated some critical features of the matured human cerebral cortex and also recapitulated the neurological pathogenesis of HSV-1 infections. We believe our acoustically assembled cortical microtissues will find wide applications in neurobiological and neurological research.

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## 3D printed pore size graded biphasic calcium phosphate scaffolds for bone tissue regeneration

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**Introduction:** Functionally graded scaffolds (FGS) are attractive in bone tissue engineering (BTE) since their mechanical properties can be tuned and they can elicit desired biological responses. The gyroid unit is currently a popular triply periodic minimal surface (TPMS) structure for BTE scaffolds because its geometrical features facilitate cell attachment and proliferation and transportation of nutrients and oxygen and avoid stress concentration. Designing and optimizing gyroid unit-based BTE scaffolds with pore size gradient is thus necessary.

**Materials and Methods:** In this study, scaffolds with uniform pore size of 400  $\mu\text{m}$  and scaffolds with graded pore sizes from the periphery (pore size of 400  $\mu\text{m}$ ) to the center (pore size of 800  $\mu\text{m}$ ) were designed. They were then fabricated via digital light processing (DLP) 3D printing using biphasic calcium phosphate (BCP) bioceramic. All designs had the same porosity (70 vol.%). A systematic comparison of mechanical properties, permeability, in vitro and in vivo biological performance between uniform scaffolds and pore size graded scaffolds was performed.

**Results:** Results showed that DLP 3D printing was capable of fabricating both uniform and graded BCP scaffolds with high accuracy. Pore size graded scaffolds exhibited better permeability than uniform scaffolds while both types of scaffolds had the same level compressive properties. Furthermore, graded scaffolds displayed enhanced cell proliferation and promotion of osteogenesis in vitro. Also, with subcutaneous implantation of both scaffolds, scaffolds with pore size gradient were beneficial for neovascularization and further improved bone formation.

**Discussion and Conclusions:** Compared to uniform scaffolds, functionally graded scaffolds showed better permeability, enhanced biocompatibility and promotion of osteogenesis in vitro, and improved new bone formation and neovascularization in vivo while their compressive properties maintained the same level. The TPMS-based gyroid-pore structures with a pore size gradient may have improved mechanical and biological performances. This study demonstrated that the mechanical properties and biological performance of BTE scaffolds could be well balanced and tuned via structural optimization and advanced 3D printing.

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## Tough-hydrogel-based tendon-mimetic niche for enhancing stem cell-mediated tendon regeneration

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**Background:** Tendon injuries, resulting from overuse or age-related degeneration, have become a prevalent clinical problem [1]. The slow healing process of tendons is attributed to insufficient cellularity and vascularity, often leading to the formation of fibrotic scarring and adhesion. To date, mesenchymal stem cells (MSCs) have emerged as a promising candidate for tendon regeneration and repair; however, the clinical application of MSC-based therapies is impeded by obstacles such as acute cell death, low functional engraftment yields, and off-target tissue formation [2, 3]. To address these challenges, there is a need to develop strategies that can precisely induce MSCs tendon-specific differentiation within a biomimetic microenvironment prior to implantation.

**Methods:** In this study, we aimed to develop a hydrogel (TenoGel) that enables biochemical and mechanical co-stimulation to establish a functional tenogenic niche for stem cell pre-conditioning and delivery (Figure a). The efficacy of TenoGel in promoting tendon healing was evaluated by: (1) in vitro characterization, which involved evaluating the effects of human adipose-derived stem cells (ASCs) tenogenic differentiation via established tenogenic markers, and (2) in vivo evaluation using a rat patellar window defect model to examine tendon healing outcomes (Figure e).

**Results:** Our results showed that TenoGel exhibited high toughness, and encapsulated human ASCs showed high viability, early spreading/elongation and fast proliferation (Figures b and c). After being stimulated by tendon-specific inductive factors and tensile loading, hASCs revealed an organized cytoskeletal structure and increased expression of tenogenic markers (Figure d). Furthermore, in a rat patellar tendon defect model, the implantation of TenoGel with rat ASCs achieved enhanced tendon regeneration, as evidenced by wavy, organized matrix structure and enhanced biomechanical features, similar to the uninjured control group (Figure f).

**Discussion and Conclusion:** Our findings suggest that the application of bioactive TenoGel can be a promising strategy for pre-conditioning and delivery of stem cells for augmented tendon repair. Further in vitro and in vivo studies will use bioinformatics analysis to investigate the interaction between biomaterial niche and stem cell lineage transition.

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## Towards large-to-massive tendon defect repair: Development of a tendon extracellular matrix-enriched, mechanically robust scaffold

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**Background:** Repair of tendon injuries poses significant clinical challenges due to intrinsic insufficient spontaneous regeneration and high mechanical demands [1]. Hence, the goal of our study is to design and fabricate a bioactive, mechanically competent hybrid scaffold that is specially intended for repair of large-to-massive tendon defect.

**Methods:** A core-shell structured hybrid scaffold (called Teno-HyS) was developed by coating “a hydrogel shell” with tendon-specific inductive factor (as biological cues) onto the mechanically robust elastomer as the “core” (for mechanical support) (Fig. 1A). A comprehensive assessment approach was applied, including: (i) *ex vivo* characterization of hybrid construct bonding integrity and mechanical property; (ii) *in vitro* cytocompatibility, proliferation, and tenogenic differentiation of human adipose-derived stem cells (hASCs) encapsulated in Teno-HyS; (iii) *in vivo* evaluation of the Teno-HyS biocompatibility in mice; (iv) and tendon healing efficacy of Teno-HyS on large-to-massive rotator cuff tendon defects in both rats and rabbits.

**Results:** Our results demonstrated that Teno-HyS (1) showed robust interface bonding between its hydrogel shell and elastomer core, human supraspinatus tendon (SSPT)-like biomechanical properties, and exceptional suture retention *ex vivo* (Fig. 1B-E); (2) exhibited cytocompatibility, proliferation, and tenogenic differentiation of encapsulated hASCs *in vitro* (Fig. 1F-I); (3) exhibited biocompatibility *in vivo* (Fig. 1J); and (4) achieved functional shoulder recovery in a rat large rotator cuff tendon defect model evidenced by gait analysis and induced over 1 cm tendon-like tissue regeneration with robust biomechanical strength in a rabbit massive rotator cuff tendon defect model evidenced by histological analysis and mechanical test (Fig. 1K).

**Discussion and Conclusion:** Our Teno-HyS scaffold demonstrates high potential for efficient repair of large-to-massive tendon defects. Future study will focus on identification of the underlying mechanism and further validation of the Teno-HyS scaffold in larger, more clinically relevant animal models.

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## Title: Functionalization, 3D bio-printing and use of stem cell-laden bio-based photo-clickable hydrogels for spinal cord injury treatment

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Category: Tissue Engineering and Regeneration

Keyword(s): neural tissue engineering, photo-clickable, 3D-printed hydrogel

Background: Spinal cord injury (SCI) can cause severe irreversible motor, sensory, and functional disorders. Designing of biomaterials that encapsulate neural stem cells (Spinal Cord Progenitor Cells-SCPCs) and mimic native neural tissue behavior has been regarded as a promising strategy to restore lost neurological functions.

Methods: In this study, we propose functionalizing chitosan and gelatin with the photo-clickable methacryl group, allowing the preparation of 3D-printed hydrogels loaded with SCPCs. The functionalized biopolymers were successfully obtained, characterized, and the degree of substitution (DS) was determined: methacrylate chitosan (ChMA, DS ~ 26 %) and methacrylate gelatine (GelMA, DS ~ 84 %). The concentration of the ChMA: GelMA and conditions of gelation were optimized. The viability and the differentiation of SCPCs loaded in hydrogel were assessed in vitro and in vivo in complete spinal cord transection rat models.

Results: 3D hydrogel structures were prepared by digital light processing enabling specific neural guide designs for the tissue regeneration process. In vitro tests showed that SCPCs can be encapsulated with high cell viability (~ 80 %) and neuronal differentiation (Fig. 1 (a)). Preliminary results after 2 weeks of in vivo implantation into the spinal cord showed that SCPCs within the 3D hydrogels were preserved, viable, and differentiated into early neurons. The hydrogel also supported the neurofilament infiltration of the host (Fig.1 (b)). Moreover, these SCPCs-loaded hydrogels did not trigger adverse scarring and inflammation around the injury site.

Discussion and Conclusion: The ability to process hydrogels in mild conditions and form a 3D hydrogel structure loaded with cells via photo-crosslinking makes these materials potentially high impact. Together, these preliminary results demonstrate the potential of these materials for neural tissue regeneration. Coupled with cell therapy, these 3D printed-SCPCs loaded hydrogel can offer a possible spinal cord injury treatment.

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## A Mechanically Reinforced Super Bone Glue Makes a Leap in Hard Tissue Strong Adhesion and Augmented Bone Regeneration

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Title: A Mechanically Reinforced Super Bone Glue Makes a Leap in Hard Tissue Strong Adhesion and Augmented Bone Regeneration

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Category: Tissue Engineering and Regeneration

**Background:** Reduced surgical trauma, sufficient space maintenance capacity of bone graft[1] and active osteogenic microenvironment are of significant importance in bone regeneration. To fulfill such requirement, this study aims to construct a super strong bone glue (L-DPZ) utilizing PVA, L-DOPA and ZIF-8[2], and to form a high strength L-DPZ-Bio-Oss composite material for regenerative guided bone augmentation.

**Methods:** The physicochemical properties of bone glue were characterized by SEM, XPS and rheological measurements. The adhesive strength was evaluated by universal mechanics testing. The in-situ fixation and the resistance to external forces of L-DPZ-Bio-Oss in wet environment were evaluated through implant exposure model and extraction alveolar ridge preservation model in pig. Biocompatibility was tested by live/dead staining, CCK-8 as well as subcutaneous implantation model. The ability to induce osteogenic differentiation of rBMSCs was detected by ALP, AR staining and qPCR. Finally, the ability to induce new bone ingrowth and its spatial-temporal sequence of L-DPZ-Bio-Oss, and the final bone augmentation effect were verified by a rabbit calvarial critical bone defect model.

**Results:** L-DPZ bone glue was successfully constructed with an adhesion strength up to 10MPa, which was far higher than that reported in literature and commercial available ones. In Bama pig models, L-DPZ anchors Bio-Oss in the surgical site and provides excellent space maintenance performance. The biocompatibility, degradability and the ability to induce osteogenic differentiation were confirmed. Micro-CT, VG staining and sequence fluorescence showed that the material could improve the performance of space maintenance and early new bone deposition by three-way osteogenic mode.

**Discussion and Conclusion:** The proposed L-DPZ super strong bone glue can effectively anchor Bio-Oss and guide bone healing as it was degraded, achieving satisfying bone augmentation effect. The study has been published as a cover article in the journal *Advanced Science*.

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## 3D Printed Gelatin/PLLA-TMC Core-shell Scaffolds with Sustained Doxorubicin and Estradiol Releases for Tumor Obliteration and Uterine Tissue Regeneration

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Title: 3D Printed Gelatin/PLLA-TMC Core-shell Scaffolds with Sustained Doxorubicin and Estradiol Releases for Tumor Obliteration and Uterine Tissue Regeneration

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Category: Design and Application of Biomaterials

**Introduction:** Uterine cancer affects the reproduction organ of women. Chemotherapy assisted by photothermal therapy (PPT) can provide effective cancer treatment. On the other hand, after killing cancer cells, uterine tissue should be regenerated at the original cancer site via tissue-engineered strategy. Sustained local release of estradiol (E2) can promote uterine regeneration. Therefore, in this study, 3D printed gelatin/PLLA-TMC ("PTMC" in short) core-shell structured scaffolds were constructed as an on-site smart drug delivery system to chronologically deliver anticancer drug and E2 biomolecules for tumor obliteration and uterine tissue regeneration, respectively.

**Materials and Methods:** Gelatin (Gel) scaffolds incorporated with doxorubicin (DOX) were 3D printed. Polydopamine (PDA) particles encapsulated with E2 were synthesized and dispersed in PTMC solution. Dried Gel scaffolds were then soaked in PTMC solution to make Gel-DOX/PTMC-PDA@E2 core-shell scaffolds. The morphology, mechanical properties, photothermal effect, drug or biomolecule release behavior, and in vitro anticancer efficiency and biocompatibility of Gel-DOX/PTMC-PDA@E2 scaffolds were investigated.

**Results:** SEM cross-sectional images showed that PTMC-PDA shell were evenly deposited onto Gel core, and the thickness of PTMC-PDA shell was affected by PTMC solution concentration and soaking time. Tensile tests indicated that wet Gel/PTMC-PDA scaffolds had similar mechanical properties as native uterine tissue. Due to PDA particles, Gel/PTMC-PDA scaffolds reached 50°C after exposed to near-infrared laser (NIR) laser. Moreover, DOX could be quickly released in 3 days. Also, E2 release could last for over 4 weeks, and its release was sensitive to environment pH. Both DOX and E2 releases could be regulated by NIR laser radiation. In vitro anticancer experiments showed that Hela cells could be almost eradicated by Gel-DOX/PTMC-PDA@E2 scaffolds actuated by NIR laser radiation. Gel-DOX/PTMC-PDA@E2 core/shell scaffolds exhibited excellent biocompatibility.

(A) Macrographic images, (B) tensile stress-strain curves, and (C) DOX release from core-shell scaffolds.

**Discussion and Conclusions:** The deposition of PTMC on Gel surface improved mechanical strength and decreased Gel degradation rate. Core-shell scaffolds could quickly release DOX in 3 days to efficiently kill cancer cells, while the release of E2 could last for more than 4 weeks to promote uterine tissue regeneration. Furthermore, DOX and E2 releases could be tuned by NIR laser radiation. The Gel-DOX/PTMC-PDA@E2 core-shell scaffolds could provide synergistic chemotherapy and PTT to effectively kill cancer cells and simultaneously promote uterine tissue regeneration. Insights have been gained for cancer therapy and tissue regeneration.



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## Cell-derived ECM hydrogel led to enhanced wound healing through the interactions with macrophages

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Category: SYIS Tissue Engineering and Regeneration

**Background:** In recent years, the role of the immune system in tissue regeneration has been highlighted. Triggering the correct immune response during skin wound treatment could greatly affect the outcome of the healing process. The current study reveals a wound dressing materials that is not only resorbable, but also able to elicit the regeneration and remodeling immune response by utilizing decellularized extracellular matrix (ECM).

**Methods:** In this study, we fabricated a human fibroblast-derived ECM based hydrogel (FDM-gel) and delivered it to a murine full thickness skin wound model. Therapeutic effects were assessed by wound closure rate, histological analysis, ELISA, western blot, and FACS of tissue samples. In vitro studies involving macrophages were also conducted to assess its interaction with FDM-gel and the role it plays on wound healing. We explored the involvement of integrin, and cytokine and MMP secretion through ELISA, western blot, and zymography analysis.

**Results:** We found that FDM-gel treatment significantly accelerated wound closure with histological findings revealing a robust hair follicle regeneration. This was paired with high levels of growth factors with positive effects on wound healing and hair follicle regeneration in the wound. FACS analysis of the regenerated skin tissue also disclosed an increase in CD206+ macrophages, an anti-inflammatory macrophage phenotype associated with pro wound healing growth factor secretion. This was later confirmed through in vitro studies that show macrophages cultured on FDM not only expressed higher CD206 genes, but it also secreted higher levels of the growth factors VEGF and bFGF. Meanwhile other cells failed to reproduce the same effect. Later, we also discovered that this effect was attenuated when macrophage-FDM interactions were hindered by the blocking of integrins. Moreover when macrophages were depleted in vivo, the therapeutic effects of the FDM-gel was not observed. Additionally, at 2 week post-treatment there were no more traces of the gel detected in the wound tissue, proving that the FDM-gel is biodegradable. Connected to this finding, we found that macrophages cultured in vitro with FDM released higher MMP-9 levels. These MMP-9 breaks down FDM to smaller particles allowing its uptake by macrophages, which then could also be connected to the modulation of macrophage to an M2 phenotype and secretion of anti-inflammatory growth factors.

**Discussion and Conclusion:** Based on our findings, we conclude that the resorbable FDM-gel can promote faster wound closure through macrophage modulation to an anti-inflammatory phenotype. This resulted in the macrophages secreting increased levels of VEGF and bFGF that contributed to the

hair follicle regeneration observed in the histological findings. Furthermore, we discovered that this effect FDM

## Multiscaled Anisotropic Heart-on-a-chip for Drug-induced Cardiotoxicity Evaluation

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### Introduction

Drug-induced cardiotoxicity (DIC) is a major obstacle to further research on the clinical application of various new drugs. It causes adverse cardiac events, such as myocardial infarction, myocardial necrosis, and even fatal arrhythmias. Current preclinical models for evaluating DIC, including in vivo animal models and in vitro cell assays, have limitations. Animal models fail to fully recapitulate human physiology, whereas traditional 2D cell cultivation cannot reflect the complex 3D cell-cell interaction as in native cardiac tissues. Bioengineered tissues have recently received more attention as promising ex vivo models for drug research. However, major barriers remain in developing an ex vivo cardiac muscle model. Furthermore, developing an ex vivo model requires incorporating engineered myocardium tissue fabrication and real-time monitoring within an integrated system, which remains a daunting challenge. Organ-on-chip (OoC) technology has recently become an increasingly popular tool in drug development, disease modeling, and biological mechanism research. However, the native myocardium contains anisotropic and multilayered cardiomyocytes (CMs) and collagen fibers, with a gradual transition in orientation that plays critical roles in the electrophysiological properties and contractile behaviors of 3D cardiac tissue. Unfortunately, most of these previous heart-on-a-chip (HoC) models can not recapitulate the 3D architectural features of the native myocardium. Hence, integrating 3D bioengineered cardiac tissues that enable biomimetic myocardial structures and properties is an urgent requirement for heart-on-a-chip development. 3D printing offers a convenient and versatile approach for preparing custom-shaped biomimetic scaffolds with complex 3D structures. However, the current 3D-printed scaffolds can hardly induce 3D cellular organization mimicking the native myocardium owing to the lack of precise microarchitecture control. Meanwhile, electrospinning is efficient for designing nanofiber scaffolds to precisely control cellular arrangement and organization as they can mimic the physical functions of the extracellular matrix (ECM). However, fabricating such a 3D anisotropic structure using only traditional electrospinning approaches remains challenging.

### Methods

In light of these shortcomings, we hypothesized that combining 3D printing and electrospinning techniques would be a creative approach for developing multiscale scaffolds, which would create a controllable 3D multilayered macroenvironment mimicking the myocardium anatomical structure and provide an aligned microenvironment as a physical cue for guiding cellular behaviors. Firstly, combined with 3D modeling technology, microscale scaffolds with different diameters and spaces were prepared by adjusting 3D printing parameters, and nanofiber films were prepared by electrospinning technology. A series of multiscale anisotropic scaffolds were prepared, and the scaffolds were characterized. Then, CMs were seeded on multiscale anisotropic scaffolds of different specifications. The effects of different scaffolds on the orientation, maturation, and synchronous beating properties of CMs were investigated by immunofluorescence staining. 3D engineered cardiac tissue was further constructed by stacking modular scaffolds layer by layer with GelMA hydrogel. Furthermore, the visualization heart-on-a-chip with circulation system was designed and prepared by integrating microfluidic technology with 3D engineered cardiac tissue. The heart-on-a-chip platform was applied to DIC evaluation with doxorubicin (DOX) and dexrazoxane (DEX) as the drug models.

### Results

In this study, we present a hybrid biofabrication method of preparing an in vitro 3D bioengineered cardiac tissue combined with 3D printing and electrospinning technology, and we further integrated it into a microfluidic chip as a heart-on-a-chip model for drug research. The 3D multiscale modular

cardiac scaffold consists of the 3D-printed micrometer-scale scaffold frames that can mimic the aligned myocardium anatomical structure and the branched-aligned electrospun nanofibers network that enables to directionally guide cellular arrangements. Such anisotropic multiscale scaffold performed the ability to improve cardiomyocyte maturation and synchronous beating behavior. More importantly, we incorporated the 3D bioengineered cardiac tissue into a microfluidic chip perfusion system to develop an anisotropic heart-on-a-chip system. The heart-on-a-chip was used as an ex vivo model for further evaluating drug-induced cardiotoxicity and cardioprotective efficacy of DOX and DEX, respectively. These results indicated that our heart-on-a-chip model developed by integrating the 3D bioengineered cardiac tissues could effectively recapitulate the clinical manifestations of drugs.

#### Discussion and Conclusion

In summary, we successfully prepare a 3D anisotropic cardiac tissue via a hybrid biofabrication method, and we further integrate it into a microfluidic chip system to develop a heart-on-a-chip model for drug research. The multiscale scaffold fabricated via 3D printing and electrospinning had controllable micro-shapes and branched nanofiber networks. To further affect the orientation and intercellular connections of the CMs, we altered the morphology and orientation of the nanofiber networks by changing the diameter and space of the 3D-printed scaffolds as collectors. It has been demonstrated our 3D multiscale cardiac scaffold showed the ability to control 3D cellular arrangements and induce synchronous beating performance of cardiomyocytes, because the 3D-printed macroenvironment was able to mimic the myocardium anatomical structure and the nanofibers microenvironment played a physical cue for guiding cellular behaviors. The multilayer bioengineered cardiac tissue was further developed by encapsulating three individual layers of multiscale scaffolds within a hydrogel shell. Our newly developed multilayer 3D scaffold promoted the maturation of aligned and elongated CMs on each layer and individually controlled the cellular orientation on different layers in a 3D environment. When integrating the multilayered bioengineered cardiac tissue into the self-designed microfluidic chip system, an anisotropic heart-on-a-chip was obtained to use as an ex vivo platform for evaluating drug-induced cardiotoxicity. Our evaluation of the cardiotoxicity of DOX and the cardioprotective effect of DEX in anti-cancer combined therapies on the heart-on-a-chip confirmed that our model could recapitulate the clinical manifestations of both drugs. The enhanced performance of CMs on the chip may be attributed to the enhanced maturation of CMs within the 3D anisotropic heart-on-a-chip. This suggests that this 3D anisotropic heart-on-a-chip is expected to provide a promising platform for early safety evaluation during drug development.

## 4D Printed and Electrospun Hierarchical Synthetic Grafts for Vasculature Repair and Regeneration

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Title: 4D Printed and Electrospun Hierarchical Synthetic Grafts for Vasculature Repair and Regeneration

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Category: Tissue Engineering and Regeneration

**Introduction:** Cardiovascular diseases (CVDs), including atherosclerosis-induced problems such as coronary heart disease and stroke, have become a major cause for fatalities, resulting in high demands for vascular grafts for treating CVDs. Autologous vessel grafts are the best but there is a lack of suitable greater saphenous veins. The demand for synthetic grafts has thus increased greatly. Commercial synthetic grafts made from PTFE or PET have been used for decades now. But they may cause side effects such as thrombogenicity and immune response.

**Materials and Methods:** In this study, hierarchical multilayered tubular grafts with good mechanical and biological performances were developed via 4D printing and electrospinning. The inner structure of tubular grafts comprised a layer of gelatin and a layer of PDLLA-co-TMC ("PTMC" in short), with each layer being electrospun fibers to form a bilayer sheet, mimicking the structure of intima. A PTMC scaffold was 4D printed on the PTMC side of the electrospun sheet, forming the media part of vasculature. The whole structure was soaked in a solution containing EDC/NHS and heparin, allowing simultaneously crosslinking of gelatin and heparin-grafting on gelatin.

**Results:** SEM examination showed that gelatin and PTMC fibers were uniformly deposited as two layers with an interlocked interface between them. The 4D printed and electrospun structures were strongly bonded due to the fusion of two structures at the interface during the deposition of 4D printed wet PTMC scaffolds on electrospun PTMC surface. Tensile testing revealed that the multilayered grafts were strong and stretchable, with mechanical properties close to those of human blood vessels. They could also self-fold into tubular shape at 37 °C due to the shape memory property of PTMC. The heparin-grafted gelatin intima made the grafts more biocompatible than those solely made from synthetic polymers. The incorporation of heparin should improve anticoagulation behavior.

**Discussion and Conclusions:** The hierarchical grafts well mimicked the structure of human artery, with electrospun gelatin, electrospun PTMC and 4D printed PTMC representing the intima, elastic lamina, and smooth muscle cell media, respectively. The electrospun structure was dense and compact, which may prevent/limit blood leakage and could promote the adhesion and proliferation of endothelial cells, thereby enhancing endothelialization. The 4D printed structure was elastic and had regular interconnected pores, which increased the stretchability of the graft and could facilitate the penetration of smooth muscle cells. 4D printed PTMC helped to change as-fabricated flat grafts into tubular shape, making them suitable for the repair/regeneration of vasculature.

**Acknowledgement:** Support by HK RGC through grants (17200519, 17202921, 17201622 and N\_HKU749/22).

## Rapid vascular inosculation via neural tissue-engineered prevascularization in vivo enhances peripheral neuroregeneration

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## Cultivation of Chicken Muscle-derived Cells using Cyanobacteria Extract for the Sustainable Food Production

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**Background:** Cultured meat represents a promising solution to address challenges associated with traditional meat production. However, the current production of cultured meat relies heavily on the consumption of substantial quantities of culture media derived from grains and animal serum. To achieve genuinely sustainable production, it is imperative to develop alternative culture media. In this study we introduce a sustainable culture meat production system by extracting nutrients from *Anabaena* sp. PCC 7120, an autotrophic cyanobacterium, and utilizing them to cultivate chicken muscle tissue-derived cells suitable for cultured meat production.

**Methods:** Nutrients were extracted from *Anabaena* sp. PCC 7120, which was cultured under continuous light exposure, using an acid hydrolysis method with hydrochloric acid. The nutrient content of the prepared extracts was assessed using a bioprocess analyzer, the Cedex Bio. Extracts with high yields were then utilized for the cultivation of chicken muscle-derived cells.

**Results:** Upon analyzing the extracts obtained from *Anabaena* sp. PCC 7120, the glucose concentration was found to be  $7316.7 \pm 924.3$  mg/L. Additionally, the evaluation of other nutrients revealed that the concentration of glutamic acid was  $477.6 \pm 115.4$  mg/L. Furthermore, it was confirmed that the cell viability recovered when the prepared extracts were added to a medium with a 50% reduction in nutrients compared to the conventional low-glucose DMEM, compensating for the deficient nutrients.

**Discussion and Conclusion:** The glucose yield of approximately 7000 mg/L obtained from *Anabaena* in this study is around seven times higher in concentration compared to the extracts obtained from eukaryotic algae under the same conditions. Most cyanobacterial species accumulate glycogen when exposed to light, which is then utilized for ATP synthesis under dark conditions. In this case, *Anabaena* sp. was cultured under continuous light exposure, suggesting that the maximum amount of glycogen was accumulated. Consequently, it is believed that the high glucose yield was achieved. Additionally, the presence of multiple constituent amino acids in proteins was observed, enabling chicken muscle-derived cells to utilize these nutrients for cultivation. These results indicate the potential of using cyanobacteria as an alternative to conventional grain-derived nutrients for culturing animal cells as a raw material for cultured meat.

**Acknowledgement:** This work was supported by Cabinet Office, Government of Japan, Moonshot Research and Development Program for Agriculture, Forestry and Fisheries (funding agency: Bio-oriented Technology Research Advancement Institution).



## Anisotropic Tissue Manufacturing by Vertical Extrusion Cryo(bio)printing

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### Introduction:

Traditional 3D extrusion bioprinting is limited by the poor mechanical properties of water-based gel bioinks, restricting printing to the X-Y plane with layer stacking in the Z direction. This study presents a new technique that utilizes cryoprotection for bioprinting, enabling direct extrusion in the vertical direction with the presence of cells. Precise temperature control is achieved using a freezing plate.

### Subject and Methods:

The anisotropic structure and various parameters of the freezing scaffold are first evaluated. Different parameter structures and complex structures are then constructed using the cryoprinting method. Multi-material, hollow, core-shell structures are achieved using microfluidic methods for 3D bioprinting. A multi-channel printing system is used to construct vertically printed structures with multiple materials. The protective effect and biological applications of cryobioink freezing are studied. Regulation of cell behavior by anisotropic structures is investigated, followed by the biomimetic construction of muscle-tendon microstructures and muscle-blood vessel microstructures.

### Results:

The cryobioprinting technique is used to create independent filamentous structures with interconnected anisotropic microchannels, exhibiting vertically aligned gradient sizes. The mechanical properties of these structures are also enhanced. Skeletal myoblasts are used to evaluate cell viability, diffusion, and alignment in 3D structures, comparing them with standard gel structures. The technique is extended to multi-material formats. Vertical 3D cryobioprinting successfully allows the creation of independent filamentous structures with anisotropic microchannels, demonstrating improved mechanical properties. Skeletal myoblasts in 3D cryogel structures exhibit enhanced cell viability, diffusion, and alignment compared to those in standard gel structures.

### Discussion:

The novel vertical 3D cryobioprinting technique proposed in this study offers higher robustness and versatility for engineering anisotropic tissue types. The technology demonstrates potential applications in interface tissue engineering, particularly in the creation of muscle-tendon units and muscle microvasculature units. Furthermore, this method can be expanded to various fields, including tissue engineering, regenerative medicine, drug discovery, and personalized therapies.

### Conclusion:

The vertical 3D cryobioprinting technique utilizing cryo bioinks showcases the ability to create independent filamentous structures with anisotropic microchannels and enhanced mechanical properties. This technique also enhances cell viability, diffusion, and alignment in engineered anisotropic tissue types. It improves the robustness and versatility of engineering anisotropic tissue types and holds promise for various applications in tissue engineering and regenerative medicine.

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## Study on biodegradable metals for the biomedical application in dentistry

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Corrosion is a special concern for metallic materials in dental implantology, because implants protrude into the oral cavity where electrolyte and oxygen compositions differ from that of tissue fluids. In addition, the pH can vary significantly in areas below plaque and within the oral cavity. To date, most of the dental implant systems are constructed from metals or alloys with high corrosion resistance. But in the recent years, biodegradable metals had been designed and applied to the dental applications, such as membrane with degradability and osteogenesis promotion for guided bone regeneration. Pure zinc membrane was developed as a pilot research in this paper. We designed three types of pure zinc membranes: pure Zn without pores, pure Zn with 300  $\mu\text{m}$ -diameter and 1000  $\mu\text{m}$ -diameter pores respectively, with pure titanium without pores as a control. The mechanical property, in vitro immersion tests and MC3T3-E1 cell viability assays were tested. Moreover, in vivo behaviors of three type zinc membranes were evaluated by using a rat calvarial critical-sized bone defect model. Furthermore, both Mg-based biodegradable metals and Zn-based biodegradable metals had been developed as bone implant potential for cranio-maxillofacial bone regeneration, not only in the form of bulk materials but also in the form of additive manufacturing porous scaffold. Interestingly, both Mg-based biodegradable metals and Zn-based biodegradable metals had been found to be antibacterial biomaterials and hold great promise for treating orthopedic infections.

## Optogenetic controlled tissue-targeting adeno-associated virus combination for osteoarthritis gene therapy and prevention

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Title: Optogenetic controlled tissue-targeting adeno-associated virus combination for osteoarthritis gene therapy and prevention

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Category: SYIS (Student and Young Investigator Section), Stem Cells and Cell-Based Therapies

Background: Osteoarthritis (OA) is a major challenge for public health and the social economy because of population aging. However, the current treatment strategies for OA are still limited to reducing weight, physiotherapy, painkillers, and anti-inflammatory drugs. Novel treatments that promote anabolism such as cartilage regeneration are needed to achieve better therapeutics. As a complex organ consisting of multiple tissues, the joint undergoes complicated pathological changes during OA progression, which means that different tissues need different interventions during gene therapy. Complicated diseases need complicated (combined) therapeutic strategies, such as inhibiting catabolic metabolism (e.g., anti-synovitis by expressing IL-1Ra, which is under clinical trials in the USA by using Adeno-associated virus (AAV) as known as Sc-rAAV2.5IL-1Ra) and promoting anabolic metabolism (e.g., promoting cartilage regeneration by activating TGF- $\beta$  pathway). To establish a combined therapeutic strategy, gene therapy is a promising strategy by using tissue-targeting AAVs to deliver different genes to different tissues and achieve different interventions. Aims: AAV is the most popular vector for gene therapy because it is non-pathogenic, safe, effective, and less immunogenic than other vectors. However, there are some drawbacks, such as the lack of targeting and neutralizing antibody interference, which limited AAV's application. Among these limitations, lack of targeting is the most important one, because a higher tissue-targeting property indicates a lower treatment dosage, lower immunoreaction, and lower costs. Thus, our first aim is to screen the novel cartilage targeting AAV (C.T.-AAV) and synovium targeting AAV (S.T.-AAV) respectively by using directed evolution technology to prepare for OA gene therapy by promoting anabolic metabolism (TGF- $\beta$  pathway for cartilage regeneration) and inhibiting catabolic metabolism (IL-1Ra for anti-synovitis). However, TGF- $\beta$  overactivity may lead to cancerization. Optogenetics provides powerful tools for light-controlled signaling pathway activity and gene expression, etc. Thus, our second aim is to spatiotemporally control the TGF- $\beta$  pathway activity by incorporating the genes of light-responsive proteins into the TGF- $\beta$  receptor genes and packing by C.T.-AAV to induce cartilage regeneration and avoid cancerization, and to construct IL-1Ra constant expression system by packing by S.T.-AAV to anti-synovitis. The comminated optogenetic TGF- $\beta$  genes will be delivered into articular cartilage by C.T.-AAV (C.T.-AAV: TGF- $\beta$ ) to realize the light-controlled ON-OFF Switch property and avoid overactivity and cancerization. After establishing the optogenetic controlled C.T.-AAV: TGF- $\beta$  system, and S.T.-AAV: IL-1Ra system, our third aim is to test the preventive and therapeutic effect of this combined therapeutic strategy in vivo in the well-established rat destabilization of medial meniscus (DMM) OA model.

Methods: Directed evolution was used to screen the novel C.T.-AAV and S.T.-AAV based on an established library of  $1 \times 10^{11}$  variants (Figure A). The optogenetic controlled TGF- $\beta$  pathway activity was molecularly built by using the blue light responded cpLID-SspB combination that incorporated to T $\beta$ RI and T $\beta$ RII (Figure H). The in vivo experiments will be performed in the DMM OA model and double-blind evaluation.

Results: The C.T.-AAV (Figure B,C,F,G) and S.T.-AAV (Figure D,E,F,G) have been successfully screened that showed specific cartilage and synovium tissue affinity respectively. The cpLID-SspB reconstructed TGF- $\beta$  pathway activity was significantly controlled by the blue lighting (Figure I-J).

Discussion and Conclusion: We wish this project will offer a new opinion on OA gene therapy and prevention, especially the application of optogenetics in traditionally challenging diseases. Moreover, we will establish a stable tissue-targeting AAV screening platform based on this project, thus, to screen various tissue-specific targeting AAVs, and this will be beneficial for the combined gene therapy strategies, and preclinical and/or clinical trials. On top of these cumulative and ongoing works, we wish to push forward the OA treatment to the precision medicine level via optimized tissue-targeting AAVs: genes system and optogenetic tools that manipulate various joint tissues respectively.

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## Viscoll Collagen Membrane For Regeneration Of Cornea: A Comprehensive In Vivo Study

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Title:

Viscoll collagen membrane for regeneration of cornea: a comprehensive in vivo study

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Category:

Design and Application of Biomaterials

Background:

Donor cornea transplantation is the most common method of surgical treatment for most diseases of the cornea; however, the global shortage of donor tissue significantly complicates its use. Thus, the development of alternative approaches based on tissue engineering and regenerative medicine is necessary to solve this problem.

Methods:

In this work, we assembled a strong, transparent, biocompatible collagen membrane by using only high concentrated collagen solution (Viscoll collagen) without chemical cross-links technique. And we performed in vitro and in vivo studies to check its effectiveness on regeneration of damaged cornea.

Results:

Mechanical properties of Vicoll collagen membrane (VCM) are comparable to those of a normal cornea. Implantation of VCM in rabbit cornea pocket resulted in an increased overall thickness of the cornea and its strength characteristics, as well as the maintenance of transparency for up to six months postoperatively [1]. In next study after removing a portion of the stroma, a VCM was implanted into the corneas of rabbits. After 6 months, the active migration of host cells into VCM was noted, with the preservation of corneal transparency in all experimental animals. Effective integration of the VCM with corneal tissue promoted nerve regeneration in vivo, as confirmed by in vivo confocal microscopy [2].

Discussion and Conclusion: We also demonstrated the safety and efficacy of the VCM for corneal stroma regeneration. In addition to its excellent optical and mechanical properties, the VCM promotes active cell migration and can be implanted into the corneal stroma using tools and techniques that mimic those used in human corneal keratoplasty. It can be concluded that the VCM has the real potential to solve the problem of the global problem of lack of donor tissue for the treatment of corneal blindness.

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## Immunological aspect of xeno-RPE sheet transplantation in a non-human primate rejection model

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Category: stem cell and cell-based therapy

Background: Localized immune rejection and inflammation response of transplanted RPE cells in humans haven't been well understood. This study aimed to investigate the immune response and survival of human induced pluripotent stem cell-derived retinal pigment epithelium (iPSC-RPE) monolayers in healthy and diseased monkey retinas without systemic immunosuppression.

Subjects and Methods: In this study, we investigated the transplantation of human iPSC-RPE monolayers into healthy and micropulse laser-induced diseased retinas in monkeys. The xenografts were surgically delivered into the subretinal space during vitrectomy surgery, and closely monitored for 12 months using multimodal ophthalmic imaging to detect any signs of rejection or inflammation. The survival of the transplanted cells was assessed through histological analysis, while pigmentation levels of the RPE cells were monitored over time.

Results: Our study demonstrated that the transplantation of human iPSC-RPE monolayers in healthy monkey retinas resulted in delayed localized inflammation and immune rejection. The immune response was observed earlier in cases where the outer retinal-blood barrier (RPE layer) was disrupted by micropulse laser. Interestingly, the immune rejection and inflammation were limited to the area surrounding the transplanted grafts, and not relevant to the surgical sequence in both eyes of the same non-human primate. Despite a gradual loss of pigmentation, the xeno-transplanted cells displayed long-term survival in the subretinal space, as confirmed by histological assessments.

Discussion and Conclusion: Our findings have important implications for the clinical application of RPE cell therapy. The delayed and localized immune response observed in our study, along with the long-term survival of the transplanted cells, provides valuable insights into the immune response to this therapeutic approach. These insights may inform the development of new strategies to enhance the safety and efficacy of RPE cell therapy in clinical trials, ultimately advancing the field and improving patient outcomes.

Acknowledgment: This work was supported by the National Research Foundation (NRF), Singapore, under its Competitive Research Programme (CRP) [NRF-CRP21-2018-00103] and National Additive Manufacturing Innovation Cluster (NAMIC), Singapore #M22N2K0007





## Injectable gel with nucleus pulposus-matched viscoelastic property prevents intervertebral disc degeneration

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### Introduction

Intervertebral disc (IVD) degeneration (IVDD) that greatly affected by regional biomechanical environment is a major cause of low back pain. Injectable hydrogels have been commonly studied for treatment of IVDD due to their capability of mimicking extracellular matrix structure to support cellular behavior and clinical prospects in minimally invasive treatment. However, most hydrogels suffer from complicated chemistry, potential uncertainty and toxicity from in-situ gelation, and mismatch with IVD mechanical environment that limit their therapeutic effects or clinical translation in IVDD or intervertebral disc defect repair. For IVD lesion repair, the study aims to develop a novel hydrogel with shear-thinning enabled injectability, high biosafety, and mechanical properties adaptable to the IVD environment, using a simple chemistry and method. And therapeutic efficacy of the novel hydrogel in the treatment of IVDD or intervertebral disc defect will be revealed.

### Subjects and Methods

A glycerol cross-linked PVA gel (GPG) was synthesized based on multiple H-bonds formation between glycerol molecules and PVA chains. The rheological and mechanical properties were tested. The swelling ratio was measured. The micro-architecture was observed through scanning and transmission electron microscopes. Nucleus pulposus (NP) cells were cultured in GPG-coated plates or silicone chambers treated under hydrostatic or dynamic loading in vitro, and examined for proliferation, vitality, apoptosis, expression of catabolic and anabolic markers. GPG was injected in needle puncture (IDD) or NP discectomy (NPD) models in vivo, and examined through magnetic resonance imaging, micro-computed tomography scanning and histological staining.

### Results

GPG had a highly porous structure consisting of interconnected pores. Meanwhile, the GPG had NP-like viscoelastic property, and was able to withstand the cyclic deformation while exhibiting a prominent energy dissipating capability. In vitro cell tests demonstrated that, the hydrogel significantly down-regulated the expression of catabolic markers, maintained the level of anabolic markers, preserved cell proliferation and vitality, reduced apoptotic rate of NP cells under pathologically hydrostatic and dynamic loading environments compared to cells cultured on untreated plate or silicone chamber. In vivo animal studies revealed that injection of GPG efficiently maintained NP structural integrity, IVD height and relative water content in IDD models, and stimulated the fibrous repair in NPD models.

### Discussion and Conclusion

Effective clinical strategy for treatment of IVDD is still lacking. This study showed that GPG, with high injectability, NP-like viscoelastic characteristics, good energy dissipating properties and swelling capacities, preserved NP cells vitality against pathological loading, and had therapeutic effects on IVD repair in IDD and NPD models. Prepared with simple chemistry and procedure, the cell/drug-free GPG with high bio-safety and shear-thinning enabled injectability bears great translational potential for the clinical treatment of IVDD via a minimally invasive approach.

## Ultrathin W<sub>O</sub>x Nanoribbons with High M2 Macrophage Induction and Antibacterial Abilities Promote the Repair of Diabetic Bone Defects

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Title: Ultrathin W<sub>O</sub>x Nanoribbons with High M2 Macrophage Induction and Antibacterial Abilities Promote the Repair of Diabetic Bone Defects

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**Introduction:** The repair of bone defects in diabetes remains a major challenge in the field of biomedicine because of the disturbance of bone immune homeostasis and the susceptibility of exposed wounds to bacterial infection. Clinically, the combination therapies of reactive oxygen species (ROS) clearance and antibacterial therapy have been widely used in the complications of diabetes mellitus. However, most of them were integrated ROS scavenging materials and antibacterial materials into a composite nanomaterial system. The complexity and instability of the material system structure limit their clinical application. In addition, although the ROS clearance property of reported nanomaterials worked well in dealing with oxidative stress, their excessive clearance of ROS usually disrupted ROS homeostasis in vivo, thus inhibiting the activation of M2 macrophages, and leading to the failure of immunoregulation therapy. Therefore, how to synergistically achieve multi-functionalization on a single nanozyme and precisely regulate the ROS content at a physiological level to better adapt to the dynamic polarization of M1/M2 macrophages for combination therapy of diabetic bone defect repair remain challenges.

**Subjects and Methods:** In this work, we used a new wet-chemical approach to construct ultrathin tungsten oxide (W<sub>O</sub>x) nanoribbon. In addition, we detected its effects on macrophage polarization, osteogenesis, and antibacterial activity in vitro and in vivo.

**Results:** Material characterization confirmed that we successfully constructed a new class of ultrathin W<sub>O</sub>x nanoribbons with high oxygen vacancies. Cell viability assays showed that W<sub>O</sub>x nanoribbons

possessed excellent biocompatibility and degraded more slowly in the acidic environment for enhancing their biosafety in diabetic therapies.

The in vitro experiments confirmed that WOx nanoribbons could remove various ROS. Moreover, 50  $\mu\text{g mL}^{-1}$  of WOx nanoribbons had the best induction effect on M2 macrophages and could also reverse the polarization M1 macrophages in a high glucose environment. In addition, metabolomic analysis indicated that WOx nanoribbons improved OXPHOS by the moderate ROS clearance, thereby efficiently promoting M2 macrophage polarization in a high glucose environment. Furthermore, WOx nanoribbons could significantly promote osteogenic differentiation of BMSCs in a high glucose environment by efficiently inducing M2 macrophage polarization. In the meanwhile, antibacterial experiments revealed that the synergistic effect of WOx nanoribbon and its photothermal property provided efficient antibacterial activity.

The in vivo evaluation in BKS-db mice showed that WOx nanoribbons could markedly facilitate the repair of diabetic bone defects infected with *Staphylococcus aureus*.

**Discussion and Conclusion:** We report a new class of ultrathin WOx nanoribbons that showed a moderate removal capability for multiple ROS, resulting in significant induction of M2 macrophages and efficient antibacterial properties under NIR for efficient combination therapy of diabetic bone defects. We found that the WOx nanoribbons could achieve the highest rate of M2 macrophage induction (86.3%) compared with those of the reported traditional molecule antioxidants and ROS scavenging metal oxide-based nanozymes used in DM. The multiple properties within a single WOx nanoribbon showed a specific advantage compared with the traditional combination therapy in managing the disordered immune homeostasis and bacterial infection in diabetes. As a result, the WOx nanoribbons significantly promoted the repair of mandibular bone defects infected with *S. aureus* in BKS-db mice. The WOx nanoribbons open up new avenues for combination therapy in tissue regeneration and exhibit promising development potential for the clinical treatment of diabetic bone defects.

## Biomimetic Marine Sponge-derived Inorganic Particle-enhanced Injectable Hydrogels for Bone Tissue Engineering Promote Bone Reconstruction

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### Background:

Bioceramics or biomimetic inorganic particles have been utilized as a promising approach for regenerating and repairing damaged bone tissue in bone tissue engineering. Marine sponge-derived inorganic particles (MSIP) are promising candidates for biomaterials in bone tissue engineering due to their unique composition and structure, which includes various elements such as biologically active silica that can support cell growth and promote bone formation and mineralization.

### Methods:

Designing an injectable hydrogel (HC-MSIP) using enzymatic cross-linking of a carrier composed of hyaluronic acid (HA) and chondroitin sulfate (CS) incorporating marine sponge-derived inorganic particles (MSIPs) and characterizing the hydrogel for its morphology and in vitro mineralization ability. Additionally, we evaluated the osteogenic activity in in vitro and in vivo mouse skull defect models.

### Results:

In this study, HC-MSIP hydrogel showed enhanced mineralization ability in simulated body fluid (SBF). In vitro, studies confirmed MSIP-dependent upregulation of bone formation markers and increased BMP-2 expression and demonstrated that MSIP induces osteogenic differentiation of tonsil-derived mesenchymal stem cells (hTMSCs) by providing a biomineralized environment. In vivo, the evaluation of a mouse calvaria defect model showed that HC-MSIP hydrogel could promote bone tissue reconstruction.

### Discussion and Conclusion:

Our study demonstrated that the HC-MSIP hydrogel can enhance bone formation in vivo, and the MSIP-dependent bio-mineralization microenvironment can promote the osteogenic differentiation of hTMSCs. These findings suggest that biomimetic marine sponge-derived inorganic particle-reinforced injectable hydrogels hold promise as potential candidates for clinical applications in bone tissue engineering and bone regeneration.

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## Effects of the differentiation state of cultivated corneal stromal keratocytes on corneal opacity resolution

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**Introduction:** The corneal stroma is populated by keratocytes (CSKs). In injuries, these cells differentiated into stromal fibroblasts (SFs) and myofibroblasts to repair the wound, often resulting in fibrosis due to the aberrant extracellular matrix (ECM) secretion. Cell therapies have recently been proposed for the treatment of corneal opacities. We have developed a protocol for the propagation of CSKs in an “activated” CSK (A-CSK) state from donor cornea tissue [1,2]. We were interested to study the therapeutic efficacies of the cultivated stromal cells in 3 differentiation states: CSKs, A-CSKs, and SFs in corneas with acute opacity.

**Subjects and Methods:** The opacity manifested 7 days following excimer laser ablation on the rat corneas. A total of 40,000 cells (CSKs, A-CSKs, or SFs) were injected into the haze region. Each group was represented by at least 6 rats. The treatment outcomes were compared to the non-treated population (control). The rats were subjected to weekly slit-lamp and in vivo confocal microscopic (IVCM) examinations, and the corneal haze was graded and quantified. The corneas were then harvested for immunofluorescence staining (IF) of keratocan, lumican, fibronectin, collagen 3A1, and  $\alpha$ -smooth muscle actin (ASMA). The collagen fiber morphometry and organization were resolved with small-angle X-ray scattering (SAXS) and transmission electron microscopy (TEM).

**Results:** The CSK injection was effective in resolving the corneal haze. At week 3 post-injection, the median haze score was 0.5 (IQR=0.375). The A-CSK injection efficacy was only marginally worse than the CSKs, with a score of 1 (IQR=0.375). In contrast, the haze persisted in the control corneas, which scored 2 (IQR=1.5;  $p=0.001$ ), and in the SFs-injected rats, which scored 2 (IQR=0.75;  $p=0.009$ ). The IVCM of the anterior third of the stroma confirmed the slit-lamp observation, where the CSKs-injected corneas showed a significantly lower haze density than the SF-injected ( $p=0.049$ ) and control corneas ( $p<0.001$ ), whereas the A-CSK administration caused a marginally worse outcome than the CSKs ( $p=0.512$ ). From IF, we deduced that the superior haze resolution in the CSKs-injected corneas was due to the absence of fibrosis markers, fibronectin, collagen 3A1, and ASMA and the restoration of proteoglycans close to the state of a naïve cornea. The corneas injected with A-CSKs and SFs expressed fibrosis markers and a lower level of proteoglycan recovery. SAXS revealed that the CSKs restored the collagen fiber organization (matrix order) to that of naïve samples ( $p=0.961$ ). On the other hand, the SFs-injected and control corneas had a significantly lower matrix order than the naïve corneas (both  $p<0.001$ ). TEM confirmed the X-ray scans, where CSK treatment resulted in a more regular alternation of the stromal lamellae, with regular fibrillar spacing.

Discussion and Conclusion: Injection of cultivated CSKs improved is a promising cell therapy for acute corneal haze. Injection of stromal cells in a more advanced differentiation state, such as the A-CSKs and SFs, did not yield similar efficacies and molecular responses. The CSKs resolved the haze by restoring the cornea's collagen fiber organization and proteoglycans. These effects were attenuated in the SF-treated corneas.

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## Cell sheet-based in vitro human liver model recapitulates hepatocellular ballooning

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Category: Tissue Engineering and Regeneration

Background: Lack of appropriate translational preclinical in vitro human models hinders the drug development for nonalcoholic steatohepatitis (NASH). As far as we know, hepatocellular ballooning which is a key histologic feature in the determination of the diagnosis of NASH has been seldom reported in in vitro models.

Methods: In this study, a cell sheet-based three-dimensional (3D) model was engineered to incorporate lipotoxic stress risk factors (high glucose, high insulin) with co-cultured primary human hepatocytes and normal human dermal fibroblasts.

Results: Enlargement of hepatocytes, loss of cytoplasmic keratin, appearance of Mallory-Denk bodies, and abundant fat droplets accumulation were observed after only a few days culture. Additionally, ultrastructural characteristic findings of induced ballooned hepatocytes (iBHs) in human NASH, including enlarged mitochondria with crystalline inclusions, dilated endoplasmic reticulum, and MDBs formation were also observed in the 3D model. Furthermore, albumin secretion was not affected in iBHs, but urea synthesis as well as cytochrome P450 enzyme (CYP) activities including CYP1A2 and CYP3A4, were significantly reduced in iBHs. Besides, loss of bile canaliculi was observed in iBHs. These findings are consistent with clinical studies of human NASH. In addition, treatment with a TGF- $\beta$  inhibitor and a semi-synthetic bile acid analogue (obeticholic acid, phase 3 trial of NASH therapy) ameliorated the histological appearance of established iBHs.

Discussion and Conclusion: This study reports in vitro production of human iBHs by using a cell sheet-based 3D model. Similar histological, ultrastructural, and pathophysiological features to human NASH are discovered in this model. In addition, this study demonstrates the priority of iBHs in recapitulating not only histology but also clinically relevant hepatic dysfunctions in human NASH and suggests TGF- $\beta$  and bile acid related signal pathway may play important roles in the formation of iBHs.

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## Interactions between the location of endothelial cells and the process of bone vascularization

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**Background:** Bone tissue is a highly vascularized tissue, and adequate blood flow supply is one of the essential factors to consider in bone regeneration. However, it is still unclear how bone blood vessel connections are structured or how growth is mediated, which makes vascularization in bones a challenging area of research that requires environmental mimicry. Therefore, in this study, human umbilical vein endothelial cells (HUVECs) and human bone marrow-derived mesenchymal cells (hBMSCs) were co-cultured to produce spheroids containing internal blood vessels.

**Methods:** In this work, we examined the difference in co-culture spheroid angiogenesis by dividing the co-culture method into core-shell and mixed forms. The core-shell form was manufactured in M2H (hBMSCs-core and HUVECs-outer layer) and H2M (HUVECs-core and hBMSCs-outer layer), while the hybrid form was manufactured by mixing hBMSCs and HUVECs at the same time. We tested the relationship between the process of vascularization formation in bone and the position of endothelial cells using a co-culture spheroid produced in three groups.

**Results:** In vitro studies have shown that the position of endothelial cells varies depending on the type of spheroid used, including H2M, M2H, and mixed spheroids. When examining the expression of VE-Cadherin to determine the difference in endothelial cell adhesion expression according to the position of endothelial cells, M2H showed the highest VE-Cadherin expression. We attempted to understand the mechanism by associating it with ROS and Rac1. In addition, the produced spheroids were induced to form blood vessels in matrigel, and the highest angiogenesis was confirmed in M2H. Results imply that there is a high possibility of bone differentiation in M2H. As a result, we plan to attempt to induce osteoblast differentiation to demonstrate the relationship between the position of endothelial cells and the process of vascularization formation in co-cultured spheroids.

**Discussion and Conclusion:** We have shown that the position of endothelial cells can vary depending on the cell seeding order, and this can lead to differences in subsequent differentiation into osteoblasts. This study has demonstrated a strategy for controlling the position of endothelial cells to prove the relationship between the process of vascularization formation in bone.

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## Graphene Oxide-modified Conductive Hydrogels for 3D Bioprinting for Neural Tissue Engineering

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Category: Design and Application of Biomaterials

**Introduction:** 3D bioprinting is a powerful platform increasingly employed in tissue engineering to produce cell-laden scaffolds for regenerating tissues such as skin, bone, and vasculature. Gelatin and gelatin methacryloyl (GelMA) blends (GG) are appealing hydrogels for making bioinks due to their distinctive advantages including similarity to the extracellular matrix and crosslinking ability. But GG hydrogels are non-conductive, which limits their applications in neural tissue engineering. Graphene oxide (GO) is biocompatible and the addition of GO in GG hydrogels can create electroactive hydrogels, expanding significantly the application scope for GG hydrogels. In this study, GG-based conductive hydrogels were developed for 3D bioprinting for neural tissue engineering.

**Materials and Methods:** GG-based conductive hydrogels (GO/GG) were formulated using gelatin, GelMA, and GO. The characteristics of GO/GG hydrogels were investigated in terms of their printability, conductivity, mechanical properties, and swelling properties. Mesenchymal stem cells (MSCs) were added to GO/GG hydrogels for making bioinks. An extrusion-based 3D bioprinter was used to process MSC/GO/GG bioinks into cell-laden scaffolds. The cell behavior within cell-laden scaffolds was studied using in vitro culture experiments.

**Results:** Results showed that the added GO improved GG hydrogel printability and mechanical properties and slightly decreased the water absorption capability. The electrical conductivity of GO/GG composite hydrogels was enhanced with an increase in GO content. As illustrated in Fig.1, MSC/GO/GG bioinks could be printed into 3D constructs with good shape fidelity. After UV crosslinking, printed structures maintained their shape integrity and supported cell growth during in vitro culture. MSCs also maintained high viability during in vitro culture.

**Discussion and Conclusions:** Gelatin is a natural hydrogel and has Arg-Gly-Asp (RGD) motifs and accessible active groups, which are beneficial for cell attachment and growth. But gelatin is rarely used alone for 3D bioprinting. GelMA is chemically modified gelatin and is photocrosslinkable. After crosslinking, GelMA can maintain the shape of the printed structure. Combining gelatin and GelMA forms GG hydrogels with good printability and crosslinking ability. The addition of GO led to GO/GG hydrogels that had good conductivity and were suitable for neural tissue engineering. The GO/GG hydrogels could be bioprinted into 3D constructs with living cells, and cells within the hydrogels showed good growth and proliferation after bioprinting. These results suggest high potential of conductive GO/GG hydrogels for 3D bioprinting in neural tissue engineering.

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## In Vivo Study of biodegradable pure magnesium membrane-guided bone regeneration

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**Background:** Magnesium metal has become a popular research topic in of orthopaedics and dental implant materials. Magnesium is a type of degradable metal with the mechanical properties of metal and biodegradability. The elastic modulus of magnesium metal is closer to the dense bone in the human body than other metal, and it can relieve the stress shielding effect better than titanium alloy and stainless steel. Magnesium ions, the degradation product of magnesium, can promote the deposition of calcium phosphate and collagen and promote the osteogenic reaction. The magnesium ions on the material's surface can promote of bone cell adhesion. Thus, magnesium and magnesium alloys are more likely to be used in guided bone regeneration membrane materials

**Methods:** Our previous research explored the problem of matching the degradation of magnesium metal with the bone formation process under the physiological conditions of specific parts (such as alveolar bone). Based on prior in-vitro experimental research, we established and maintained a bone regeneration space by preparing high-purity magnesium membranes. In vivo animal experiments were conducted to explore the effect of pure magnesium membrane combined with bone graft material to repair vertical bone defects in dog mandibles.

**Results:**At 4, 12, and 24 weeks after surgery, the relative bone mineral density of the pure magnesium membrane group was higher than that of the collagen membrane and the control group. At 24 weeks after surgery, the pure magnesium membrane was completely degraded; the osteogenesis area was filled with new bone, and the trabecular bone was thick and dense. No inflammation was observed in the epithelial layer or the mucosa's lamina propria.

**Discussion and Conclusion:** The pure magnesium membrane can guide and control the outline of the regenerated bone, which can better to maintain the bone regeneration space in some larger bone defects, and has good biocompatibility with the local soft tissue in the implanted area.

**Acknowledgement:** Supported by the Academic Research Projects of Beijing Union University ( No. ZK70202006 )

## Stem Cell-Recruiting Injectable Microgels for Repairing Osteoarthritis

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**Introduction:** The differentiation potentials and viability of stem cells are often impaired during cell isolation and delivery.

**Subjects and Methods:** Inspired by the phenomenon where islands can recruit seabirds for nesting, “cell island” microgels (MGs), that is, growth factor-loaded methacrylated hyaluronic acid and heparin blend MGs, which can recruit endogenous stem cells and promote chondrogenic differentiation, are constructed using microfluidic technology and photopolymerization processes, followed by non-covalently binding platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-beta3 (TGF- $\beta$ 3).

**Results:** The loading efficiency of PDGF-BB and TGF- $\beta$ 3 are 96% and 91%, respectively. In vitro and in vivo experiments find that the “cell island” MGs can enhance the migratory capacity of cells and recruit them from their niche via releasing PDGF-BB. Meanwhile, by using hyaluronic acid, the “cell island” MGs provide a suitable microenvironment for cell attachment and spreading. Furthermore, the “cell island” MGs induce chondrogenic differentiation of the recruited cells via releasing TGF- $\beta$ 3 and present a promising therapeutic effect for osteoarthritis.

**Discussion and Conclusion:** In sum, this developed “cell island” MG might serve as a temporary “nest site” to allow the migration, adhesion, and differentiation of endogenous stem cells, which can be a promising candidate rather than the conventional cell-seeded scaffolds for promoting tissue regeneration.

## MiR-181b Inhibitor Enhanced the Reparatory Effect of E11 via ECM Remodeling in Dental Pulp

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Title: MiR-181b-2-3p Inhibitor Promotes Reparatory Effect of E11 on Extracellular Matrix Remodeling of Dental Pulp

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Category: Stem Cells and Cell-Based Therapies

**Background:** The extracellular matrix (ECM) plays an important role in human dental pulp cell (hDPC) proliferation, differentiation, and repair. However, the research on epigenetic regulation of ECM remodeling in order to promote dental pulp repair is still limited. Enhancer of Zeste Homolog 2 (EZH2) is a regulator of pulp inflammation, and E11—a selective EZH2 inhibitor—can inhibit pulp ECM degradation. In this study, the reparatory role of E11 on inflammatory pulp tissue was further studied, and a microRNA regulation was used to promote the reparatory effect of E11 on pulp tissue.

**Methods:** HDPCs were treated with miR-181b-2-3p mimics/NC or inhibitors/NC, and the expression levels of ECM-related factors were assessed by qPCR and Western Blot experiments. Subsequently, the targeted inhibition effects of miR-181b-2-3p on the 3' UTR regions of target genes were verified by dual-luciferase reporter experiments. HDPCs were stimulated with 2  $\mu$ Mol/L E11 and 50 nM miR-181b-2-3p inhibitors, and the cell migration ability and ECM-related factors expressions were later evaluated. The rat pulp inflammation model was used as an *in vivo* experiment model to observe the reparatory effect of E11 and miR-181b-2-3p inhibitors on the inflamed pulp tissue, and the outcomes were analyzed by H&E staining, Masson staining and immunohistochemical staining.

**Results:** The protein expression levels of TGF- $\beta$ 2 and FNDC5 were significantly downregulated in hDPCs after transfection with miR-181b-2-3p mimics ( $P < 0.01$ ), whereas the mRNA levels of TGF- $\beta$ 2 and FNDC5 were significantly upregulated after transfection with miR-181b-2-3p inhibitors ( $P < 0.05$ ). Dual luciferase reporter assays demonstrated that miR-181b-2-3p targets FNDC5 and TGF- $\beta$ 2. The migration rate of hDPCs was strongly enhanced when E11 was used with miR-181b-2-3p inhibitor, compared to E11 was used alone ( $P < 0.05$ ), and the protein levels of COL1A1, FN1, and TGF- $\beta$ 2 were significantly upregulated in hDPCs when E11 was used with miR-181b-2-3p inhibitor ( $P < 0.01$ ). The results of animal experiments showed that miR-181b-2-3p inhibitors acting alone or in combination with E11 both exhibited certain reparatory effects on inflamed pulp tissue.

**Discussion and Conclusion:** This study demonstrated the potential of E11 to promote the inflamed pulp tissue repair. A miR-181b-2-3p inhibitor was able to promote the reparatory effect of E11 on pulp tissue via ECM remodeling.

**Acknowledgement:** This work was supported by National Natural Science Foundation of China 81800959.

## Atlas of in situ stem cells recruited by 3D printing tissue engineering graft for skull defect full regeneration

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**Background:** Current treatments for critical-sized bone defects often lead to incomplete repair or non-union, and full-thickness defects of the calvarium still present reconstructive challenge. The transplantation of in vitro engineered bone grafts incorporating stem cells have shown limited potential for further clinical applications, due to long-time of culture and limited in vivo survival rate.

**Methods:** In this study, we designed a strategy of in situ expansion of specific stem cell subpopulations for bone regeneration through transplantation of a neurotrophic supplements (NSs)-incorporated 3D printing hydrogel graft. By single-cell RNA analysis, we show that a unique atlas of in situ stem/progenitor cells that was generated by the NSs-incorporated graft during calvarial bone healing.

**Subjects and Results:** Full-thickness regeneration of rat critical-sized calvarial defects was successfully achieved by the in situ culture system with NSs. Notably, we found a local expansion of resident Msx1+ skeletal stem cells (SSCs) after transplantation of the in situ cell culture system. Moreover, the enhanced calvarial bone regeneration is accompanied by an increased endochondral ossification that closely correlated to the Msx1+ SSCs, whereas the calvarium is a well-known site of intramembranous ossification.

**Discussion and Conclusion:** Our results on the in situ skeletal stem cells that expanded by a specific tissue engineered graft during bone regenerative process could provide a rational cellular basis for the efficient regeneration of large bone defects. In summary, our findings illustrated the time-saving strategy and regenerative efficacy of in situ culture system that targeting major cell subpopulations in vivo for rapid bone tissue regeneration.

**Acknowledgements:** This work was supported by the National Key Research and Development Program of China (2017YFA0104900) and NSFC Grant (NO. T2121004).

## Coadministration of Hyaluronic Acid-Modified Liposomes with Hydrogel Microneedles Enhances the Efficacy of Fisetin against Skin Aging

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**Introduction:** The production and accumulation of senescent cells contribute to the occurrence and progression of skin aging. Fisetin was the most potent senolytic in cultured senescent human skin fibroblasts (HSFs) due to its capacity for senescent cell elimination[1]. The efficient delivery of fisetin to skin cells for anti-aging therapy is an emerging challenge. The hydrogel microneedles (MNs) with advanced properties and targeting liposome carriers inspired the design of the anti-skin aging therapy.

**Subjects and Methods:** In this study, we designed and assembled biodegradable microneedles with silk fibroin methacryloyl and recombinant human Collagen XVII methacryloyl hydrogel (SFMA/rhCol17MA hydrogel). Furthermore, fisetin was carried by the hyaluronic acid-modified liposome (HA-Lip), aiming to precisely and effectively act at CD44+ cells and locally applied with the hydrogel microneedles. The safety and efficacy in ameliorating skin aging of this approach with fisetin loaded were tested in vitro and in vivo.

**Results:** The microneedles of SFMA/rhCol17MA hydrogel with or without HA-Lip@Fis were successfully synthesized and assembled with excellent mechanical behaviors and good biocompatibility. This application exhibited the sustainable release and efficient delivery of fisetin which attenuated inflammatory factors and oxidative stress during skin photoaging in cell and tissue levels. It rejuvenated UVA-irradiated HSFs with suppressed ROS production and senescent-associated secretory phenotype (SASP). The efficacy against skin aging in vivo was further demonstrated with improved skin moisture, elasticity, thickness, and collagen contents.

**Discussion and Conclusion:** This study reported a safe, minimally invasive approach involving the HA-modified liposomes and SFMA/ rhCol17MA hydrogel MNs to achieve the efficient transdermal delivery of fisetin. We demonstrated that its application exerts anti-aging efficacy in vitro and in vivo, providing a strategy for future anti-skin aging therapy.

**Reference:**

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**Acknowledgment:** This work was supported by Sun Yat-Sen University-Yixian Group (YSG) Joint Laboratory for Precision Skin Health Research



## Gelatin microporous annealed particle scaffolds for human spinal cord progenitor cell delivery

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Title: Gelatin microporous annealed particle scaffolds for human spinal cord progenitor cell delivery

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Category: SYIS (Student and Young Investigator Section), Tissue Engineering and Regeneration

Background: Spinal cord injury (SCI) may result in the irreversible impairment of the sensory, motor and autonomic functions. Correspondingly, cells encapsulated in bulk hydrogels have been used to replace the lost neural cells. However, the lack of topographical cues and porosity has led to non-aligned axonal regeneration, as well as ineffective cell migration and growth.

Methods: In this study, we integrated human induced pluripotent stem cells derived spinal cord progenitor cells (SCPCs) in gelatin microsphere slurry that can facilitate freeform printing and form microporous annealed particle scaffolds by ruthenium-catalyzed photocrosslinking.

Results: The gelatin microsphere slurry exhibited shear-thinning property and self-recovery behavior that allowed freeform printing of sacrificial Pluronic F127 inks. Ruthenium-catalyzed photocrosslinking of the tyrosine groups in the gelatin and removal of the sacrificial ink by dissolution in 40°C led to channel formations in the microporous annealed particle scaffold. In vitro studies showed that the micropores support SCPC viability and differentiation along the neural lineage with longer neurite extensions. Additionally, the microchannels were able to facilitate aligned axonal regeneration.

Discussion and Conclusion: Our study has demonstrated that gelatin microspheres were able to form microporous annealed particle scaffolds with channels that resulted in enhanced SCPC survival, differentiation with extended neurites and axon alignment. The scaffold exhibited its potential as a promising method for stem cell delivery and neural tissue construct fabrication to treat SCI.

# DLP Fabricating of Precision Hierarchical Porous Composite Scaffold for Bone Tissue Engineering Application

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Title: DLP Fabricating of Precision Hierarchical Porous Composite Scaffold for Bone Tissue Engineering Application

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Category: Design and Application of Biomaterials

## Introduction

The composition and structure of biomaterial scaffolds are particularly important for the biological effects of bone repair. The hierarchical structure of bone provides valuable inspiration for the design of bone repair materials to enhance bone regeneration.

## Subjects and Methods

In order to prepare macro/micro/nano hierarchical porous gelatin methacryloyl (GelMA)/silk fibroin methacryloyl (SilMA)/hydroxyapatite (HAp) scaffold (GSH scaffold): 3D printing was used to prepare the macro structure, aqueous two-phase lotion method was used to prepare micropores, and photocrosslinking was used to form hydrogels to form nano pores. The effects of scaffolds on cell behavior and bone regeneration were tested in vitro and in vivo.

## Results

The addition of polyethylene oxide provided (PEO) approximately 42 $\mu$ m micropores for GSH composite inks. The inks had good rheological properties and the 3D-printed accuracy of about 105  $\mu$ m. In vitro experiments showed that GSH scaffold can significantly promote the adhesion and proliferation of osteoblasts, and promote cell migration into the scaffold. In vitro osteogenic differentiation experiments showed that GSH scaffolds significantly promoted the osteogenic differentiation of BMSCs. In vivo experiments of rabbit skull defects, GSH scaffolds can significantly promote the regeneration of new bone, but only a small amount of bone tissue was found in scaffolds without micropores.

## Discussion and Conclusion

We demonstrated that 3D printing combined with PEO, can integrally and accurately prepare hierarchical porous scaffolds, significantly promoting cell adhesion, proliferation, migration, differentiation and new bone regeneration. This research provided a new method for 3D printing integrated preparation of hierarchical porous scaffolds and a clue for the differential mechanism of bone regeneration of the scaffold microstructure.

## Morphological analysis of senescent cells for label-free monitoring in mesenchymal stem cells

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**Introduction:** Mesenchymal stem cells (MSCs) possess immunosuppressive properties and the ability to differentiate into various mesenchymal tissues, making them highly valuable for regenerative medicine applications. However, their stable expansion culture remains challenging due to the rapid loss of proliferative capacity and cellular functionality during continuous passages, often attributed to cellular senescence induced by stress during passage. To address this challenge, our group has been developing a non-destructive and early prediction method for MSC quality using image-based analysis combined with machine learning [1, 2]. While we have previously demonstrated "over-passage-related cell quality decay" using morphological profiles, it was unclear whether we could predict different types of senescence.

**Subjects and Methods:** In this study, our objective was to identify distinct types of senescence in MSCs through the utilization of our morphology-based cell quality evaluation technology.

Furthermore, to improve the sensitivity of our morphological profiling, we endeavored to detect stressed MSCs at the single-cell level, given the significant heterogeneity inherent in MSC populations. MSCs were subjected to diverse senescence-inducing stresses, including over-passage and exposure to senescence-inducing drugs, and their morphological data was captured using an automated image acquisition system during the course of culture. The obtained time-course images were then analyzed to extract morphological features using image processing techniques, which were subsequently utilized for clustering and machine learning analyses.

**Results:** Our results revealed two significant insights: (1) senescent cells exhibit different morphologies depending on the senescence cascade, and (2) senescence-related sub-populations can be detected and profiled by our morphology-based image cytometry analysis.

**Discussion and Conclusion:** Our study reveals that despite the existence of various senescence-related phenotypes, our morphology-based cell quality evaluation technique that does not require labeling can assess the status of MSCs solely from their microscopic images. Therefore, our cell quality evaluation technology holds great promise in enabling a detailed cell quality assessment in the context of MSC-based cell therapies and cell manufacturing.

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### Acknowledgement

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## GDNF-loaded Polydopamine Nanoparticles-based Anisotropic Scaffolds Promote Spinal Cord Repair by Modulating Inhibitory Microenvironment

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**Introduction:** Spinal cord injury (SCI) is among the most devastating central nervous system insults that disrupt spinal cord function and its anatomical structure. After SCI, the oxidative stress induced by excessive reactive oxygen species (ROS) often leads to prolonged neuroinflammation that results in sustained damage to the spinal cord tissue. The success of SCI repair lies in the proper modulation of the pathophysiological process that provides a favorable microenvironment for neuronal survival and axon growth. With the rapid development of tissue engineering scaffolds, modulation of the microenvironment of the injured spinal cord has become possible, which may achieve better functional recovery in SCI repair. Polydopamine (PDA) shows remarkable capability in scavenging ROS to treat numerous inflammatory diseases. The ultimate goal of SCI repair is to promote the recovery of its motor function. Neurotrophic factors are the most promising candidates that facilitate neuron survival, and axon regeneration. In contrast to other neurotrophic factors, Glial cell-derived neurotrophic factor (GDNF) could significantly promote the survival of motor neurons, showing good treatment potential for the locomotor recovery of SCI.

**Methods:** In this work, we developed the mesoporous PDA NPs (mPDA NPs)-embedded anisotropic gelatin composite scaffold (PDA/Gelatin), which guides neuronal axons to grow in a directional arrangement, and effectively scavenges ROS to promote M2 polarization of microglia in the injured spinal cord, thereby inhibiting local inflammation response to provide a favorable microenvironment for SCI repair. In addition, we encapsulated GDNF into mPDA NPs to release drugs intelligently under the acidic microenvironment of SCI, thus facilitating nerve repair.

**Results:** The GDNF@PDA/Gelatin scaffold could reduce intracellular ROS, decrease inflammation by promoting M2 microglia polarization. The elimination of intracellular ROS may be attributed to improvements in the intrinsic antioxidant capacity. Moreover, local sustained delivery of GDNF under the acidic microenvironment of SCI was also realized. The GDNF@PDA/Gelatin scaffold exhibited excellent repair capabilities of SCI, preventing host cells from apoptosis, inducing axons regeneration to form new synapses with target neurons, and enhancing the recovery of motor function.

**Discussion and Conclusion:** We developed a GDNF-loaded PDA/Gelatin composite scaffold to promote SCI repair. mPDA NPs in the scaffold eliminate intracellular ROS and regulate microglia polarization, which reduces inflammation responses and nerve cell apoptosis of the damaged spinal cord. Furthermore, GDNF released from the scaffold enhances axons regeneration of CST to grow over the lesion site and form new synapses with downstream target cells, thus enhancing motor function recovery. Therefore, local modulation of the inhibitory microenvironment and combination with the delivery of neurotrophic factors may have potential clinical applications for the SCI treatment.

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## Metabolic Reprogramming for Attenuating Inflammatory Bone Loss: The Potential of Magnesium-Based Biomaterials

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Title: Metabolic Reprogramming for Attenuating Inflammatory Bone Loss: The Potential of Magnesium-Based Biomaterials

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Category: Design and Application of Biomaterials

Background: Access inflammation negatively affects the skeletal system, leading to bone loss and delayed fracture healing. Magnesium-based biomaterials have gained recognition in orthopedics for bone regeneration via mobilizing endogenous stem cells. However, their roles in inflammatory bone loss and the mechanism of immune metabolism regulation require further investigation.

Methods:

We compared the effects of different metal ions on cell survival and investigated the regulatory effects of magnesium ion on major cell populations during bone remodeling under normal and endotoxin (LPS) -induced hyperinflammation. We established a mouse model of inflammatory bone loss to investigate the feasibility of magnesium intake to alleviate bone loss and the regulation of stem cell survival, macrophage immune regulation, and osteoclastogenesis. We also designed an intelligent magnesium ion release biomaterial to test its role in the healing process of inflammatory fractures.

Results:

Our results showed that magnesium and calcium ions have excellent biocompatibility among metal materials commonly used in orthopedics. Magnesium ions significantly promoted stem cell survival and macrophage M2 polarization, especially under inflammatory conditions, which is related to the regulation of magnesium ion on mitochondrial energy metabolism homeostasis. Moreover, magnesium ion significantly reduced the mitochondrial metabolism level of osteoclasts and inhibited osteoclast formation under inflammatory conditions. In vivo, intake of magnesium ion significantly alleviated bone loss caused by hyperinflammation. The effect of magnesium ion on bone remodeling cell population was consistent with that in vitro. Accordingly, we designed an intelligent inflammation responsive magnesium ion release system, which has a positive therapeutic prospect for facilitating fracture healing under inflammatory conditions.

Discussion and Conclusion:

Our findings further highlight the potential of magnesium-based biomaterials in bone diseases by demonstrating their role in managing inflammatory bone loss and revealing their metabolic regulation mechanisms. The intelligent inflammation-responsive magnesium-releasing material we developed may help treat bone loss under access inflammation.

## Development of Freeze-dried Lipid Nanoparticles Entrapped mRNA for Long Term Storage under Mild Condition

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### • Introduction

Gene therapy has emerged as a popular treatment for several diseases. Lipid nanoparticles (LNPs) are a promising safe and noninvasive delivery vehicle for gene therapy. The LNP entrapped mRNA (LNP-mRNA) vaccines (Comirnaty and Spikevax) have an enormous contribution to suppress the SARS-CoV-2 pandemic recently. However, the LNP-mRNA is not stable at room temperature for long term storage. Additionally, the molar ratio of each lipid composition in the LNP-mRNA can have a significant impact on their performance, especially PEGylated lipid, which is commonly used to avoid aggregation of LNP-mRNA. While most researches are focused on optimizing the stability of LNP-mRNA through cryoprotectants, our study aims to optimize the stability of LNP-mRNA by optimizing the ingredients of LNPs at mild condition (4 degree). We have adopted an I-optimal mixture design to determine the optimal molar ratio of four types of lipids (ionizable lipid, cholesterol, phospholipid, and PEGylated lipid) that can achieve long-term storage under mild condition (4 degree).

### • Methods

We used an I-Optimal Mixture Design as the Design of Experiment (DoE) to establish 15 different LNP-mRNA (eGFP) variations with varying molar ratios of LNPs. This design allowed us to screen the optimal formulation and to establish the relationship between the transfection efficacy and the ratio of each component. After the screening of DoE, we further tested the stability of LNP-mRNA that have high transfection efficiency of mRNA, in which the ratio of SM-102 (ionizable lipid) and DMG-PEG2000 were fixed and the ratio of DSPC and cholesterol was modified. The encapsulation efficiency, size, and the transfection efficiency of mRNA on LNP-mRNA were characterized after lyophilization and stored at 4 °C for 1, 4, 8, 16 weeks respectively. The encapsulation efficiency of mRNA was evaluated using RiboGreen reagent, and the transfection efficiency of mRNA was evaluated using flow cytometry after mRNA-LNP was transfected into HEK-293T cells.

### • Results

Based on the results of I-Optimal designed screening, we developed a model that can describe the relationship between mRNA expression and the ratio of each component in LNP-mRNA (coefficient of determination ( $r^2$ )=0.87). In terms of predicting the relationship between the molar ratio of each lipid component and mRNA expression after transfection, we observed that DMG-PEG2000 had a highly negative effect on the amount of mRNA expression after the transfection. Conversely, SM-102 had a positive effect to enhance expression of mRNA. In the stability experiment, We found that the LNP-mRNA complex had the highest stability when the ratio of DSPC to cholesterol was 0.36.

### • Discussion and Conclusion

It was possible to find optimal lipid composition of LNP-mRNA that exhibits high stability and high transfection efficiency by modifying the molar ratio of the lipid components. The positive effect of SM-102 on transfection is explained as its ability to facilitate endosomal escape after the LNP-mRNA is transfected into the cell. Regarding the stability, the optimal ratio between DSPC and cholesterol is an important factor because they are the primary lipid components on the surface of LNP-mRNA. The stiffness of the surface of LNP-mRNA may protect the degradation of LNP-mRNA with aging.

## Human Pluripotent Stem Cell Culture and Differentiation into Retinal Pigment Epithelium on Poly(vinyl alcohol-co-itaconic acid) Hydrogels Grafted with Several Designed Peptides

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### Introduction

Age-related macular degeneration (AMD) is one of the major causes of blindness [1]. The characteristic of AMD is progressive loss of retinal pigment epithelium (RPE). Human pluripotent stem cells (hPSCs) derived RPE is an ideal source of regeneration therapy of AMD patients. However, current approaches for culturing and expansion of hPSCs rely on xeno-containing materials and have batch-to-batch variability. Therefore, we developed poly(vinyl alcohol-co-itaconic acid) (PV) hydrogels grafted with peptides to culture and differentiate hPSCs into RPE [2].

### Experiments

PV hydrogels grafted with designed peptides were prepared. X-ray photoelectron spectroscopy (XPS) was used to confirm that the peptides were grafted on the PV hydrogels for hPSCs cultivation. The expression of pluripotent markers (SSEA-4, OCT4, SOX2, and Nanog) and RPE markers (ZO-1, MITF and RPE65) of hPSCs and hPSC-derived RPE cultured on different peptides grafted PV hydrogels were evaluated by immunostaining and/or flow cytometry to investigate the optimal cell culture biomaterials for hPSC differentiation into RPE. Finally, hPSC-derived RPE was transplanted into RCS rats subretinally for preclinical safety and efficaciousness study.

### Results and Discussion

hPSCs can attach to PV hydrogels with high expansion folds and retained their pluripotency. Compared to commercial xeno-containing Matrigel, PV hydrogels grafted polypeptides manifested lower adhesion effect during the process of differentiation of RPE, which may be caused by the less cell binding sites. High purity of pigmented cobblestone-like RPE cells were successfully differentiated from hPSCs on Matrigel. We expect to optimize the novel PV hydrogels which may induce better hPSC expansion and higher differentiation efficacy of hPSCs into RPE and support clinical trials of usage of hPSC-derived RPE in future.

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## Label-Free Single Cell Impedance Analysis of iPSC-derived Spinal Cord Progenitor Cells for Rapid Profiling of Safety and Efficacy Phenotypes

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Category: SYIS (Student and Young Investigator Section) Enabling Technologies

Background: The use of induced pluripotent stem cells (iPSCs) to derive progenitor cells for transplantations has garnered huge interest because of its strong potential to repair damaged tissue, especially after spinal cord injuries. However, the differentiation of iPSCs into neurons and neural cell types have been highly variable and requires reliable assessment of its differentiation efficiency, to validate both its safety and quality. Phenotyping is often performed via label-based methods including immunofluorescent staining or flow cytometry analysis. These approaches are often expensive, laborious, time-consuming, destructive, and severely limits their use in large scale cell therapy manufacturing settings. On the other hand, cellular biophysical properties have demonstrated a strong correlation to cell state, quality and functionality and can be measured with ingenious microfluidic label-free technologies in a rapid and non-destructive manner.

Methods: Herein, we report a single-cell, high-throughput, label-free impedance biophysical cytometry for detection of several key iPSC and neural stem and progenitor cell (NSC/NPC) phenotypes. Briefly, human iPSCs were differentiated into spinal cord progenitor cells (SCPCs) and the impedance signatures of both these cell types measured using a microfluidic impedance cytometry chip. Multi frequency impedance signals were employed to probe and measure biophysical properties including cell size, deformability, membrane opacity and nucleus opacity. Furthermore, we implemented a multi-dimensional data reduction technique using Uniform Manifold Approximation and Projection (UMAP) to analyse and visualise 9 different impedance-based parameters.



Results: We found that mean impedance signatures of cell size, deformability, membrane opacity and nuclear opacity for iPSCs were distinctively different from SCPCs. This was further demonstrated by analysis of their biophysical properties on the UMAP which displayed a distinct separation of these cell types into 2 populations. Additionally, artificial spiking of iPSCs in SCPCs at 1%, 5% and 10% concentrations demonstrated the capabilities of our impedance UMAP plot to accurately quantify the presence of iPSCs at these different concentrations. We also negatively affected the differentiation process of iPSCs to SCPCs by adjusting the concentration of CHIR, a Glycogen Synthase Kinase-3 $\beta$  (GSK-3 $\beta$ ) inhibitor that is responsible for neural differentiation efficiency. Immunofluorescence staining assays revealed that a decrease of CHIR during the differentiation process caused a decrease in SCPC phenotypes whilst having an increase in iPSC phenotypes. More importantly, impedance profiling of these cells and quantification of their phenotypes using the gating strategy of both iPSCs and SCPCs exhibited a clear trend on the UMAP that was consistent with both immunofluorescence staining and flow cytometry analysis.

Discussion and Conclusion: Impedance profiling of iPSCs and SCPCs has clearly indicated its capabilities to identify and quantify key phenotypes of iPSCs and SCPCs for end-point validation of safety (tumorigenicity) and quality (spinal cord repair) parameters. Our process is label-free and the lack of any chemical or biological sample processing renders the cell functional and viable for subsequent downstream analyses. Thus, our technology has a strong potential to be developed as a versatile in-line process analytical tool for cell therapy manufacturing to assess and validate iPSC related cell therapy products for safety and quality.

## Evolution from Bioinert to Bioresorbable: In Vivo Comparative Study of Additively Manufactured Metallic Bone Scaffolds

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**Introduction:** Additive manufacturing (also called 3D printing) techniques have been utilized and developed in the field of orthopedic implants. When an implant aims for bone regeneration, the porous structure will be beneficial for mass transfer and cell migration, leading to enhanced angiogenesis and new bone growth. Even though many bioabsorbable materials, including polymers and ceramics, have been applied in 3D-printing scaffolds, their mechanical strength is still considered insufficient, especially in the case of load-bearing applications. Bioabsorbable metals, such as magnesium (Mg) and zinc (Zn), have shown great potential as the next generation of biomaterials thanks to their decent mechanical properties, biodegradation, and biocompatibility. Here, we use additive manufacturing to fabricate porous Mg and Zn scaffolds. Their degradation behavior and the interaction between bone tissue and scaffolds are studied in vivo with a rabbit femur model.

**Subjects and Methods:** Mg and Zn scaffolds are composed of WE43 and Zn1Mg alloys, respectively. Titanium (Ti) scaffolds made of Titan-Grade 1 are used as a benchmark group. Laser Powder Bed Fusion (L-PBF) is facilitated for the manufacturing of different scaffolds. The scaffold is in the shape of a cylinder with a diameter and height of 4 mm. The pore size of all scaffolds is around 250  $\mu\text{m}$ . Two circular defects with a diameter of 4 mm are created by the drill in the distal femur, and then two scaffolds made of the same material are inserted into these two defects separately. Two collection time points are set to 5 and 25 weeks. Systematic toxicity is evaluated by checking the blood and internal organs. Collected femur bones with scaffolds are analyzed with Micro CT scanning, SEM/EDX, nanoindentation, histochemical and immunohistochemical staining, and finite element analysis (FEA). **Results:** (Toxicity) No systematic toxicity is found for all scaffolds based on the analysis of blood and internal organs. (Degradation) The degradation behavior is inhomogeneous. Different sections of the Mg scaffold show various degradation tendencies. Regions exposed to the bone defect have fewer oxides compared to the regions exposed to the marrow cavity. The regional divergence of degradation is less significant in Zn scaffolds. (Bone regeneration) The new bone growth displays various behavior in different stages. In the early stage (week 5), no mature cortical bone can be found at either end of the scaffold. However, in the late stage (week 25), more new growth bone is distributed in the Mg scaffold exposed to the defect area, and a compact bone layer is formed around this region, which can be confirmed by Micro CT and staining images. Zinc scaffolds also demonstrate a similar growth pattern. On the contrary, in the case of Ti scaffold, no compact bone layer is formed around scaffolds. The detailed protein expression related to osteogenesis, immune response, and angiogenesis around Mg/Zn/Ti scaffolds is analyzed through IHC staining. The protein expression is rated and mapped according to regions. (Mechanical analysis) Nanoindentation results demonstrate that the compression modulus of bones shows various values in different regions. In the end, FEA is performed on models derived from the Micro CT, involving bones and scaffolds. FEA results show the distribution of loading stress and visualize the stress-shielding effect from scaffolds, which is closely related to bone regeneration.

**Discussion and Conclusion:** Mg and Zn scaffolds present promising biodegradation behavior and biological response. The ingrowth of new bones shows different patterns on Mg, Zn, and Ti scaffolds, leading to the process of bone defect healing. The protein expression analysis and FEA provide mechanism insight into the interaction between the bone and the scaffold.

## Exosomes Derived from CD271+CD56+ Bone Marrow Mesenchymal Stem Cell Subpopulation Identified by Single-Cell RNA Sequencing Promote Axon Regeneration after Spinal Cord Injury

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**Introduction :** Spinal cord injury (SCI) results in neural tissue damage. However, the limited regenerative capacity of adult mammals' axons upon SCI leads to persistent neurological dysfunction. Thus, exploring the pathways that can enhance axon regeneration in injured spinal cord is of great significance.

**Subjects and Methods:** Through the utilization of single-cell RNA sequencing in this research, a distinct subpopulation of bone marrow mesenchymal stem cells (BMSCs) that exhibits the capacity to facilitate axon regeneration has been discovered. Subsequently, the CD271+CD56+ BMSCs subpopulation was isolated using flow cytometry, and the exosomes derived from this subpopulation (CD271+CD56+ BMSC-Exos) were extracted and incorporated into a hydrogel to create a sustained release system. The aim was to investigate the therapeutic effects of CD271+CD56+ BMSC-Exos and elucidate the underlying mechanisms involved in promoting axon regeneration and neural function recovery.

**Results:** The findings indicate that CD271+CD56+ BMSC-Exos share similar physical and chemical properties with conventional exosomes. Importantly, in an SCI model, in situ implantation of CD271+CD56+ BMSC-Exos within the hydrogel matrix resulted in increased expression of NF and synaptophysin, markers associated with neuronal differentiation and synaptic formation, respectively. This intervention also contributed to improved neural function recovery. In vitro experiments demonstrated that CD271+CD56+ BMSC-Exos treatment significantly enhanced axon extension distance and increased the number of branches in dorsal root ganglion axons. Moreover, further investigation into the molecular mechanisms underlying CD271+CD56+ BMSC-Exos-mediated axon regeneration revealed the crucial involvement of the miR-431-3p/RGMA axis.

**Discussion and Conclusion:** In summary, the in situ implantation of CD271+CD56+ BMSC-Exos presents a promising and effective therapeutic approach for SCI.

## Increased extracellular vesicles release of transplanted neural stem cell via HIF-1 $\alpha$ /Rab17 pathway enhances neurofunctional recovery of spinal cord injury

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**Background:** As a severe traumatic disease of central nervous system, spinal cord injury (SCI) causes damage of individual health and increase of medical economic burden. Cell transplantation is a very promising therapy for spinal cord injury. Neural stem cells (NSCs) transplantation aims to promote the directional differentiation of NSC into neurons, replacing damaged axon and synapse. But this process is limited by the poor microenvironment of SCI. The therapeutic mechanism of NSCs transplantation remains unclear.

**Methods:** We cultivated neural stem cells from fetal Nestin-Cre/Rosa26-TdTomato mice in vitro. By transplanting this neural stem cells with fluorescence to the injury site of mice spinal cord resection model, we captured the release of small extracellular vesicles (sEVs) of transplanted NSCs and tested the functional recovery of SCI.

**Results:** The fluorescence lineage tracing showed that most of the transplanted neural stem cells differentiated into astrocytes, rather than neurons. The transplanted NSCs released substantial sEVs under hypoxic microenvironment. Neurons, endothelia and macrophages in the injury site engulfed NSCs-sEVs, which improved axon regeneration, angiogenesis and immune regulation. Multi-omics and CHIP-PCR identified that HIF-1 $\alpha$ /Rab17 pathway mediated the sEVs release of hypoxic NSCs. Proteomics revealed the complicated protein-protein interaction network of NSCs-sEVs to the recipient cells.

**Discussion and Conclusion:** Hypoxia is a classical factor promoting release of extracellular vesicles. Transplanted NSCs promoted axon regeneration, angiogenesis and immune regulation by releasing substantial sEVs via HIF-1 $\alpha$ /Rab17 pathway under hypoxic condition. This revealed a novel mechanism of neural stem cell transplantation and provided new therapeutic targets for SCI treatment.

## Exosomal miR-34a-5p derived from EGFR-positive neural stem cells promoted neurite regrowth and functional recovery after spinal cord injury by targeting HDAC6

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**Background:** Spinal cord injury (SCI) causes severe axon damage, usually leading to permanent paraparesis. Despite many strategies dealing with SCI, there is still a lack of effective regenerative therapy. Recent studies have suggested that exosomes (Exos) derived from neural stem cells (NSCs) may hold promise as attractive candidates for SCI treatment. After injury, the endogenous NSCs of a heterogeneous nature are activated and express EGFR named EGFR+NSCs, which could migrate toward the lesion site. However, the role and mechanisms of EGFR+NSCs derived exosome-mediated neurite regrowth, as well as their therapeutic effects for promoting traumatic spinal cord injury repair, are unclear.

**Methods:** In this work, we extracted the exosomes derived from the EGFR+NSCs and evaluated their effects on SCI treatment. Using miRNA-seq and Dual-Luciferase Reporter Assay, we recognized the functional cargo and its downstream pathway in vitro and in vivo.

**Results:** In this study, we successfully isolated exosomes derived from the EGFR+NSCs and discovered that local application of EGFR+NSCs-Exos can effectively promote neurite regrowth in the injury site of spinal cord-injured mice and improve their neurological function recovery. Using the miRNA-seq, we characterized the microRNAs (miRNAs) cargo of EGFR+NSCs-Exos and identified a neuroprotective miR-34a-5p which was highly enriched in EGFR+NSCs derived exosomes. And using the Dual-Luciferase Reporter Assay, it was discovered that miR-34a-5p can inhibit the function of HDAC6 by binding to its mRNA, which contributes to microtubule stabilization and autophagy induction for SCI repair.

**Discussion and Conclusion:** Our research demonstrated a novel therapeutic approach to improving hindlimb function by activating endogenous NSCs to secrete exosomes and provide a precise cell-free based treatment strategy for SCI repair.

## Targeted Delivery of RGD-CD146+CD271+ Human Umbilical Cord Mesenchymal Stem Cell-Derived Exosomes Promotes Blood Spinal Cord Barrier Repair After Spinal Cord Injury

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**Background:** Spinal cord injury (SCI) is a prevalent form of central nervous system injury, and effective treatment methods are currently lacking. The preservation of blood-spinal cord barrier (BSCB) integrity is crucial in SCI treatment. Identifying a subpopulation of exosomes with stable BSCB function and achieving specific targeted delivery could serve as an alternative therapeutic approach for SCI.

**Methods:** Flow cytometry was utilized in this study to isolate specific subpopulations of CD146+CD271+ UCMSCs, from which engineered exosomes (RGD-CD146+CD271+ UCMSC Exos) with targeted neovascularization function were obtained through gene transfection. In vivo and in vitro experiments were conducted to investigate the targeting and therapeutic effects of RGD-CD146+CD271+ UCMSC Exos, as well as to explore the potential mechanisms underlying BSCB stabilization and neural function recovery.

**Results:** RGD-CD146+CD271+ UCMSCs Exos exhibited similar physical and chemical properties to conventional exosomes. Notably, following intranasal administration, RGD-CD146+CD271+ UCMSC Exos exhibited enhanced aggregation at the SCI center and demonstrated specific targeting of neovascular endothelial cells. In the SCI model, intranasal administration of RGD-CD146+CD271+ UCMSC Exos reduced Evans blue dye leakage, increased tight junction protein expression, and improved neurological function recovery. In vitro testing revealed that RGD-CD146+CD271+ UCMSC Exos treatment significantly reduced the permeability of bEnd.3 cells treated with OGD, restoring the integrity of tight junctions. Moreover, further exploration into the molecular mechanism underlying BSCB stabilization by CD146+CD271+ UCMSC Exos identified the crucial role of the miR-501-5p/MLCK axis in this process.

**Discussion and Conclusion:** In conclusion, the findings of this study provide evidence that RGD-CD146+CD271+ UCMSC Exos possess the capacity for specific targeting of miR-501-5p to vascular endothelial cells in the SCI epicenter. This targeted delivery effectively suppresses the expression of MLCK, resulting in reduced disruption of tight junctions, enhanced stabilization of the BSCB, and ultimately facilitates neurological recovery in mice. These results offer novel insights and establish a theoretical foundation for the treatment of SCI.

## Targeted transplantation of engineered mitochondrial compound promotes functional recovery after spinal cord injury by enhancing macrophage phagocytosis

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**Background:** Mitochondrial transplantation has shown promise in treating ischemic and hypoxic diseases. However, its potential application for acute spinal cord injury (SCI) remains to be explored. Local injection of mitochondria into the injured spinal cord can further damage the fragile neural tissue, and the effect of transplanted mitochondria on cells and the microenvironment within the injured area is still largely unknown. Following SCI, macrophages infiltrate to clear waste such as myelin debris through phagocytosis. However, when the amount of waste exceeds the phagocytic capacity of macrophages, it deposits at the injury site and builds up inside cells. This, in turn, can lead to mitochondrial dysfunction and inflammation in macrophages, ultimately hindering neural tissue regeneration. Therefore, enhancing phagocytosis and promoting the phenotypic transformation of macrophages by mitochondrial transplantation hold great significance.

**Methods:** In this study, we developed an engineered mitochondrial compound (Mito-Tpp-CAQK) to target the injured spinal cord with a peptide sequence cations-cysteine-alanine-glutamine-lysine (CAQK) and triphenylphosphonium cations (Tpp), using IL-10-induced Mertk-high-expression (Mertkhi) bone marrow-derived macrophages as energetic sources of mitochondria. We tested its effects on macrophage phagocytosis, cellular inflammation, tissue repair, and functional recovery of mice with SCI.

**Results:** We verified that IL-10 induced Mertkhi macrophages have an enhanced mitochondrial function, and this enhancement is associated with the upregulation of Mertk expression. Therefore, we utilized them as donor cells for mitochondrial transplantation. We have successfully constructed an energetic mitochondrial compound suitable for mitochondrial transplantation. The intravenously administered mitochondrial compounds effectively targeted the injured epicenter of the spinal cord and were mainly taken up by macrophages. Furthermore, we confirmed that mitochondrial compounds enhanced macrophage phagocytosis of myelin debris, reduced mitochondrial dysfunction, and alleviated the inflammatory response of macrophages both in vitro and in vivo. Finally, the mitochondrial compounds promoted tissue repair and functional recovery in mice after SCI.

**Discussion and Conclusion:** This study provides a novel approach for mitochondrial transplantation in SCI by modulating macrophage phagocytosis and phenotypes. Intravenous transplantation of Mito-Tpp-CAQK led to better tissue repair and functional recovery after SCI in mice. Overall, this therapeutic approach shows great promise for clinical applications.

## Constructing an ACL reconstruction graft Containing a Sleeve-shaped Bone Tunnel Filler with Chondrogenic Inducibility for the Enhancement of Reconstructed Graft-to-Bone Integration: An Application Study of Novel Linking-peptide to Tether Exosomes on a Collagen scaffold

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**Background:** As the population of sports enthusiasts increases, anterior cruciate ligament (ACL) tear has become one of the most common knee injuries. Due to poor regenerative potential of ACL, surgical reconstruction with graft is always necessary, especially when the ACL is absolutely teared. However, the reconstructed graft usually integrated to bone tunnel by a layer of disorganized fibrovascular scars, which is structurally and compositionally inferior to native ligament-to bone interface, as known as "entheses". As a consequence, this fibrovascular scars cannot endow the reconstructed ACL firmly attaching to bone tunnel, which may further cause failure of graft implantation and require additional revision surgery. Hence, developing some adjuvant strategies to realize rapid and strong integration of the reconstructed ACL to bone tunnel is meaningful and urgent. This study breaks through the traditional single-phase structure design of ACL graft, and intends to construct a novel ACL reconstruction graft containing a sleeve-shaped peri-graft filler in bone tunnel. At the same time, exosomes that contribute to cartilage differentiation are innovatively combined with the graft through "linking peptides", so as to prolong the local action time of exosomes and improve the chondrogenic induction activity of the graft. This provides a new design for engineering ACL reconstruction graft.

**Methods:** a sleeve-shaped cartilage scaffold was designed and evaluated, which was obtained by a novel acellular method using costal cartilage. A novel chondrogenic stem progenitor cell subset was isolated from adipose tissue, and exosomes of the subsets were extracted and their functions were studied. Through software analysis and experimental verification, the "linking peptide" that can bind to CD9 on exosome surface and collagen in the sleeve-shaped cartilage scaffold is screened out. By constructing a reconstruction model of ACL injury in Beagle dogs, we explored the repair effect of functional optimized cartilage layer scaffolds and studied the related mechanisms.

**Results:** The "sleeve" scaffolds with well-preserved extracellular matrix obtained by experiments showed good cytocompatibility and no obvious cytotoxicity. The exosomes of adipose-derived chondrogenic stem progenitor cell subsets can promote chondrogenic differentiation of stem cells. By combining the selected "linking peptide" with collagen, the function of the "sleeve" cartilage scaffold is effectively improved, and the repair effect of anterior cruciate ligament injury reconstruction in Beagle dogs is effectively promoted. Meanwhile, it has been shown that exosomes of chondrogenic stem progenitor cell subset induce chondrogenic differentiation of synovial mesenchymal stem cells by activating CREB and inhibiting classical Wnt/ $\beta$ -catenin.

**Discussion and Conclusion:** We successfully constructed "sleeve" scaffolds with optimized cartilage layer function, which can promote the enhancement of reconstructed graft-to-bone integration, and has the advantages of high new bone density, new fibrocartilage layer, good biomechanics, and low immunogenicity; Exosomes can effectively promote the integration of reconstructed graft to bone tunnel mainly by activating CREB and inhibiting classic Wnt/ $\beta$ -catenin to induce chondrogenic differentiation of synovium mesenchymal stem cells, while promoting stem cell migration to the injured site.



## Autocrine COL-RECM of meniscus fibrochondrocytes promotes tissue regeneration of meniscus implants

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Title: Autocrine COL-RECM of meniscus fibrochondrocytes promotes tissue regeneration of meniscus implants

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Category: Tissue Engineering and Regeneration

Background: Meniscus tear is one of the most common sports injuries within the knee joint. Meniscus in adulthood lacks blood supply, making it difficult to self-repair after injury. Meniscectomy is inevitable for severe meniscus tears, which can disrupt joint mechanics and increase the incidence of osteoarthritis (OA). The state-of-art tissue engineering meniscus (TEMs) are composed of polymeric compound and allogeneic biomaterials, which still lack of biocompatibility and have the potential risk of disease transmission.

Methods: In this work, we developed a made-to-order cell culture medium to incubate meniscus fibrochondrocytes (MFCs) to produce extracellular matrix rich in many kinds of collagen (COL-RECM) and cell migration-related proteins (MRPs). The effect of COL-RECM on MFCs migration was evaluated in vitro. COL-RECM was then combined with PCL for the implantation. Tissue regeneration and cartilage protection induced by COL-RECM-PCL scaffold were evaluated in vivo.

Results: Under the synergistic effect of collagen and MRPs in the COL-RECM, MFCs were sequentially induced to migrate into the RECM. After implantation of composite COL-RECM-PCL scaffolds into rabbit knee joint, long-term meniscus regeneration and cartilage protection were accomplished.

Discussion and Conclusion: We presented an autocrine COL-RECM to promote the MFCs migration and meniscus reconstruction. This kind of meniscus repair biomaterial – COL-RECM was extracted and amplified from autologous meniscus tissue, which presents superior biocompatibility and avoids the risk of disease transmission, and can also serve as an injectable biomaterial to promote meniscus repair in the future. The composite COL-RECM-PCL scaffolds balanced mechanical strength and biocompatibility, possessing high clinical promotion potential, which will fill the huge demand gap for meniscus defect repair materials.

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## Spheroid on-demand printing and drug screening of endothelialized hepatocellular carcinoma model at different stages

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Title : Spheroid on-demand printing and drug screening of endothelialized hepatocellular carcinoma model at different stages

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Category: Enabling Techniques

Background: Hepatocellular carcinoma (HCC) poses a significant threat to human health and medical care. Its dynamic microenvironment and stages of development will influence the treatment strategies in clinics. Reconstructing tumor-microvascular interactions in different stages of the microenvironment is an urgent need for in vitro tumor pathology research and drug screening. However, the absence of tumor aggregates with paracancerous microvascular and staged tumor-endothelium interactions leads to bias in the antitumor drug responses.

Methods: Herein, a spheroid-on-demand manipulation strategy was developed to construct staged endothelialized HCC models for drug screening. Pre-assembled HepG2 spheroids were directly printed by alternating viscous and inertial force jetting with high cell viability and integrity. A semi-open microfluidic chip was also designed to form a microvascular connections with high density, narrow diameter, and curved morphologies.

Results: Endothelial cells in the semi-open chips presented continuous growth and abundant junctions and finally reproduced the microvascular morphology with suitable diameter and vesicular structures in the HCC lesions. This research further developed a spheroid-on-demand manipulation technique to construct staged endothelialized HCC models for in vitro drug screening. The adopted AVIFJ technique was proven to have good controllability in spheroid printing, enabling spatial and temporal arrangement of spheroids inside the gel with a high survival rate and structural integrity. Discrete and fused spheroids were printed into rich endothelialized connections to biomimetic single or multi lesions in HCC stages I and II, respectively. Fluorescent staining and HE sections presented high morphological similarity to HCC tissue on the dense aggregation of tumor mass and the proximal surrounding of endothelial cells. Further, a migrating stage III HCC model preliminarily showed spheroid dispersion under the induction of TGF- $\beta$  and verified the occurrence of EMT at the protein and gene expression levels. Finally, on anti-HCC drug testing, the multi-lesion stage model was found to have stronger drug resistance, while the migrating stage had a more rapid drug response.

Discussion and Conclusion: This model construction technique achieves the controllable arrangement of tumor spheroids with the microvascular connections, addressing the spatial demands of paracrine and temporal demands of tissue development. The established endothelialized tumor model reproduces the characteristic staged activities and is expected to provide a reliable experimental platform for the future development of anti-tumor therapeutic strategies in subsequent clinical trials. The corresponding work provides a widely applicable method for the reproduction of tumor-microvascular interactions at different stages and holds great promise for the study of tumor migration, tumor-stromal cell interactions, and the development of anti-tumor therapeutic strategies.

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## Transformation of arginine into zero-dimensional nanomaterial endows the material with antibacterial and osteoinductive activity

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**Introduction:** Orthopedic implant-associated infections remain a big clinical challenge that requires extensive surgical interventions and long-term antibiotic therapies. *Staphylococcus aureus*, one of the main causes of bone infections, can delay the bone healing process and lead to bone loss. The main reason why various studies have failed to achieve both antibacterium and tissue regeneration simultaneously is that these materials eliminate bacteria by directly up-regulating reactive oxygen species (ROS) levels. However, they are generally toxic to both bacteria and mammalian cells because of the intrinsic inability of ROS to distinguish bacteria from mammalian cells. Because of the moderate positive charge, arginine CDs (Arg-CDs) efficiently disrupted bacterial membranes while promoting typical mammalian cell growth by modulating ROS levels. Therefore, Arg-CDs may be an ideal candidate for promoting infectious bone repair because of their differential regulating capacity of antioxidant enzymes between cells and bacteria.

**Methods:** In this study, we synthesized Arg-CDs by a simple pyrolysis method. To release Arg-CDs in a well-controlled way in the acidic environment of infectious bone injury, the Arg-CDs were mixed with aldehyde hyaluronic acid (HA-CHO) and gelatin methacryloyl (GelMA, G), which then formed a CD/HA/GelMA composite hydrogel (CHG) as a result of the photocrosslinking of GelMA and Schiff base interaction between Arg-CDs and hydrogel. In addition to the characterizations of the morphology and release behavior of Arg-CDs, the antimicrobial and osteoinductive activity of the composite hydrogel were evaluated *in vitro*. After implanting composite hydrogel into infectious rat femoral defects, its ability to promote bone formation and eliminate bacteria was assessed *in vivo*. Last, the underlying mechanism of osteogenesis induced by the composite hydrogel was explored.

**Results:** The developed CHG composite hydrogels can release Arg-CDs in response to the acidic bone injury microenvironment. The free Arg-CDs could selectively kill bacteria by generating excessive ROS. Furthermore, the Arg-CD-loaded HG composite hydrogel showed excellent osteoinductive activity through inducing the M2 polarization of macrophages by up-regulating interleukin-10 (Il10) expression. Together, our findings revealed that transformation of the arginine into zero-dimensional Arg-CDs could endow the material with exceptional antibacterial and osteoinductive activity, favoring the regeneration of infectious bone.

**Discussion and Conclusion:** In conclusion, we successfully fabricate zero-dimensional Arg-CDs and engineered CHG composite hydrogel that can collapse as well as intelligent controlled release of Arg-CDs in the acidic infectious bone injury microenvironment. The released Arg-CDs significantly eliminate bacteria by producing excessive ROS and promote cell proliferation by up-regulating the expression of antioxidantases. Moreover, the obtained Arg-CD-based composite hydrogel up-regulates the expression of Il10, which fuels the osteogenesis by promoting M2 macrophage polarization. Owing to the perfect combination of excellent antibacterial activity and bone formation, our CHG composite hydrogel exhibits a highly attractive application prospect in the treatment of infectious bone repair, which notably improves the success rate of bone implants.

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## Construction of decidual tissue using endometrial cell sheets and maintenance of the tissue using a perfusion bioreactor

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### Introduction

Infertility has significant negative social impacts in advanced nations. The birth rate is severely declining in these countries therefore new fertility treatment methods are urgently needed. The problems associated with existing treatments are that the mechanism of the implantation remains largely unknown, and the success rate of treatment is currently low. Therefore, we are trying to decipher the mechanism in vitro using cell sheet engineering. By layering the endometrial sheets, we could create a three-dimensional endometrium-like tissue with sufficient thickness to allow for sufficient penetration of fertilized eggs and to reproduce the unique two-layer structure of the endometrium seen in vivo. In this presentation, we will report on experiments inducing and maintaining the essential cell transformation for the process of implantation, decidualization.

### Subjects and Methods

The uterus was removed from female rats by primary and the cells were isolated by trypsin treatment. We then separated epithelial and stromal cells using static separation and formed them into sheets using the appropriate culturing method. The collected sheets were stacked in the order of stromal and epithelial layers to construct a three-dimensional endometrium-like tissue. In order to maintain the three-dimensional tissue, we performed culturing in a perfusion bioreactor with microfluidic channels. A culture medium rich in female hormones was used to induce decidualization, and the tissues were cultured for a total of 7 days from the time of sheet formation. The tissues were evaluated not only by histological evaluation but also by qPCR to determine the expression of the decidualization marker, IGFBP-1.

### Results

The three-dimensional endometrial-like tissue constructed by stacking epithelial and stromal cell sheets could be maintained using a perfusion bioreactor. We also observed that the addition of female hormones and cAMP in culture media in abundance induced morphological changes as observed by histological evaluation, and decidualization as suggested by evaluation of gene expression.

### Discussion and Conclusion

In future studies, we aim to observe the implantation process by placing fertilized eggs in the three-dimensional decidualized tissue. Additionally, we would like to evaluate the efficacy of potentially new fertility treatment, i.e., transplantation of the three-dimensional tissue in which the fertilized egg has been implanted in infertile rats.

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## Multi-material bio-printing of biomimetic interfaces using silk-based bio-inks for nasal osteochondral tissue engineering.

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Title: Multi-material bio-printing of biomimetic interfaces using silk-based bio-inks for nasal osteochondral tissue engineering.

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Category: Tissue Engineering and Regeneration. SYIS (Student and Young Investigator Section).

Background: Interface Tissue Engineering (ITE) is gaining increasing importance with increased understanding of the complex interaction between different tissues in maintaining distinct tissue function, which is especially true of the osteochondral interface. In particular, the nasal osteochondral interface poses additional structural challenges for ITE methods due to the spatially complex interface between the nasal cartilage and underlying bone structures. Bio-printing is an emerging Tissue Engineering tool that offers the capability to precisely place cell-encapsulated bio-inks to re-capitulate structurally complex tissues such as that of the nasal tissue. Yet, current bio-printing methods are lacking in its ability to generate continuous, biomimetic gradients similar to native tissues. Developing a multi-material bio-printing method that could fabricate continuous interface gradients which possesses similar interface length as that of native tissue would thus better recapitulate the bio-mechanical environment that cells experience, especially those residing near to the interface.

Methods: In previous work, we developed a multi-material extrusion bio-printing method which demonstrated the capability to bio-print interface length similar to that of native tissue (at  $457.64 \pm 136.37 \mu\text{m}$ ) and that nozzle design (specifically inlet contact angle) affects the produced interface length. We also demonstrated that mechanical stiffness of a silk-based bio-ink (known as SG-bio-inks) could modulate porcine nasal chondrocyte (pNC) and porcine calvaria osteoblast (pCO) associated ECM production. In this work, we combined both findings to develop bio-printed constructs with continuous cellular gradient to analyse the effect of mechanical and cellular gradients on embedded cell phenotype expression. Primary pNCs were encapsulated in medium stiffness SG bio-inks (Young's Modulus  $43.10 \pm 1.216 \text{ kPa}$ ) and primary pCOs were encapsulated in high stiffness SG bio-inks (Young's Modulus  $96.49 \pm 15.71 \text{ kPa}$ ) respectively and were each loaded onto two modular mixing nozzles (with 90o and 150o designs). A G-code that was obtained from STL scans of the nasal cartilage cross section was generated and used with the loaded nozzles to fabricate the nasal cartilage construct with its associated underlying osseous junction. Green and Red cell tracers were used to visualize encapsulation of pNC and pCO respectively to observe presence of cellular gradients. Bio-printed constructs were cultured for up to 14 days and evaluated using Immunohistochemistry (IHC) to determine the presence of associated phenotype markers (blue for Col I and red for Col II). Results: Similar to earlier work, the length of the interface of bio-printed constructs from 90o nozzles was significantly longer than that of the 150o nozzle, resulting in steeper gradients in constructs from the 150o nozzle. Additionally, G-Codes generated from STL scans of the nasal cartilage cross section could be used in conjunction with the bio-printing method to generate bio-printed nasal cartilage constructs with its associated osseous structures, with the 150o nozzle demonstrating more defined interfaces. IHC analysis indicate that for constructs printed with 90o and 150o nozzles, Col II presence was detected at 7 days. But by 14 days, Col II was not present in 90o bio-printed constructs whereas constructs bio-printed with 150o nozzles show similar levels of Col II staining as compared to 7 days. Col I staining was detected at all time points for all constructs.

Discussion and Conclusion: This project demonstrated that cellular gradients could be generated using the bio-printing method, while also demonstrating the ability to precisely place the interface at its designed spatial positions. Similar to previous findings, the length of the cellular gradient can be

modulated by nozzle inlet contact angle, thus allowing for one to vary interface gradient. This project also showed that gradient length of bio-ink composition play important roles in modulating embedded cellular phenotype, particularly that of nasal chondrocytes. Chondrocytes are known to de-differentiate and experience hypertrophy when cultured under conditions that are not favorable, producing Col I instead of its associated Col II phenotype expression. This could be due to an interplay between mechanical and cellular gradients present. Since osteoblasts can secrete growth factors (such as BMPs), this could have an impact on other embedded cells (such as chondrocytes) in its surrounding environment. Different interface gradients could thus indicate that there was various diffusivity of these associated growth factors which then modulates chondrocyte phenotype expression.

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## Utilizing Single-Walled Carbon Nanotubes for the Topical Delivery of Tyrosinase: A Novel Approach to mitigate Photo-Induced Skin Damage

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**Introduction:** The skin serves as the primary barrier protecting the body from various forms of damage. Ultraviolet (UV) irradiation induces inflammation and the generation of reactive oxygen species (ROS), while also triggering the degradation of the extracellular matrix (ECM) through UV-induced matrix metalloproteinases (MMPs), resulting in photo-aging. Tyrosinase, a key enzyme in melanin biosynthesis can oxidize phenol to catechol or quinone, activating phenol-containing ECM components like collagen. Therefore, delivering tyrosinase, more than 30kDa, through the dense skin layer can promote melanin formation for UV protection and densify the ECM. This approach represents a promising strategy to mitigate UV damage and enhance skin health.

**Methods:** In this study, we synthesized tyrosinase-conjugated single-walled carbon nanotubes (SaTy-SWNTs) and utilized a reverse electrodialysis (RED) system for their delivery. We assessed the UV protection ability of melanin formed by SaTy-SWNTs and observed the reduction of wrinkles both *ex vivo* and *in vivo*.

**Results:** We screened proper functionalized SWNT to find suitable size and zeta potential for skin penetration. Skin penetration ability were detected with Raman spectra and lipid-conjugated SWNT (Lipid-SWNT) were selected for suitable candidate. SaTy-SWNT showed effective tissue penetration and melanin formulation, which were observed by extracting melanin and Prussian blue staining. Crosslinking ECM were confirmed by polarized light, FT-IR spectra of collagen with or without SaTy-SWNT. We observed UV protection ability with SaTy-SWNT confirmed by H&E staining and MTC stained skin tissue on balb/c mice under high dose of UV irradiation. Moreover, increased adhesiveness of *ex vivo* porcine skin with SaTy-SWNT also confirmed the crosslinked ECM.

Furthermore, under low dose of UV irradiation of balb/c mice showed significant decrease area of wrinkle with denser ECM, which confirmed by MTC stained tissue and polarized image of ECM.

**Discussion and Conclusion:** Our study demonstrates the ability of SaTy-SWNTs to promote melanin formation within skin tissue and provide UV protection. Furthermore, SaTy-SWNTs exhibit the capacity to increase collagen density and crosslink ECM components enzymatically, effectively mitigating photo-aged skin and wrinkles. These findings highlight the potential of utilizing single-walled carbon nanotubes for the targeted delivery of large proteins with tissue-penetrating abilities, representing a novel and promising approach for applications in the field of tissue engineering.

**Acknowledgement:** This study was carried out with the support of 'R&D Program for Forest Science Technology (Project No. "2021405B10-2223-0101)' provided by Korea Forest Service(Korea Forestry Promotion Institute).



## Pilot Clinical Trial Investigating the Effectiveness of Bioinductive Scaffold Augmentation During the Repair of Acute Achilles Tendon Ruptures with Pre-existing Tendinopathy

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Pilot Clinical Trial Investigating the Effectiveness of Bioinductive Scaffold Augmentation During the Repair of Acute Achilles Tendon Ruptures with Pre-existing Tendinopathy

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### Introduction:

Achilles tendinopathy affects both active and sedentary individuals, with around 10% of individuals who have experienced Achilles tendon rupture reporting a history of pre-existing Achilles tendinopathy. Managing the surgical treatment of Achilles tendon rupture along with chronic tendinopathy poses a challenge because of the mechanical deficiencies in a weakened tendon. The use of a bioinductive collagen scaffold patch to augment tendon repair has proven to be a promising solution, demonstrated through its facilitation of healing in the repair of massive rotator cuff tears. This study aims to investigate whether implementing this approach for the augmentation of the repair in Achilles tendons with pre-existing tendinopathy will yield positive results.

### Method:

This is a case series involving patients with an Achilles tendon rupture associated with underlying tendinopathy. They underwent surgical repair, which was augmented with the use of a bioinductive collagen patch. The primary focus of the study is to document any adverse events or complications, as well as assess the patients' functional scoring by using the Foot and Ankle Outcome Score. Additionally, secondary outcomes involve measuring tendon thickness and assessing intratendinous vascularity via the modified Ohberg score using ultrasound technology.

### Results:

This review series included five patients, consisting of four males and one female with an average age of 42.1 years old (ranging from 18 to 77 years old). The average follow-up time was 4.1 months. Two out of the five patients had a tendon rupture at the Achilles insertion and the remaining three patients sustained a midportion Achilles rupture. All but one patient showed excellent surgical wound healing with no reported adverse events or complications. However, one patient with underlying diabetes experienced postoperative wound dehiscence, which required repeated debridement and delayed wound closure for proper healing. At the 6-week follow-up, the average FAOS for symptoms 62±13, pain 79±19, ADL 73±5, sports 47±20, and QOL 42±9. Ultrasonographic thickness of the repaired Achilles was 14.0±6.6 mm, compared to 7.1±1.3 mm on the non-injured side. For intratendinous vascularity, two patients received a modified Ohberg score of 2+, while the remaining two received a score of 3+.

### Conclusion:

To the best of our knowledge, we have conducted the very first study involving the use of this bioinductive implant in the Achilles tendon. In the immediate aftermath of surgery, all five Achilles tendon rupture patients with pre-existing tendinopathy who had their tendon repair augmented by this bioinductive collagen patch achieved favourable functional outcomes. This surgical approach seems to be safe and technically practical, but it warrants further investigation to determine its long-term impact on tendon biology. Large-scale prospective trials will bring clarity to this matter.

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## Recombinant Human Collagen Ameliorates Structural Damage And Dysfunction Of Skeletal Muscle After Hindlimb Ischemia

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Title: Recombinant human collagen ameliorates structural damage and dysfunction of skeletal muscle after hindlimb ischemia

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Category: Tissue Engineering and Regeneration

**Introduction:** Peripheral arterial disease (PAD) is a chronic vascular dysfunction of extremity, usually manifested as vascular blockage and accompanied by ischemic myopathy. Protecting physiological structure and function of skeletal muscle after hindlimb ischemia is the key to reduce the morbidity and mortality of PAD. Collagen type I and type III are two major ECM components that are intrinsically involved in skeletal muscle development and regeneration.

**Subjects and Methods:** In this work, a mouse model of hindlimb ischemia was established and the ischemic tibialis anterior (TA) muscle was treated by intramuscular injection of recombinant human collagen type I (rhCol I) and type III (rhCol III) matrices to investigate the protective effect of rhCol.

**Results:** Muscle contractile tests showed a significant loss of TA muscle strength after hindlimb ischemia, while both rhCol I and rhCol III treatments significantly improved muscle strength. Functional performance of skeletal muscle by a rotarod test showed that the mice after hindlimb ischemia exhibited poor exercise endurance, while the rhCol-treated mice exhibited a remarkable exercise endurance. TEM showed disorganized sarcomeres after hindlimb ischemia, while rhCol-treated myofibers exhibited healthy appearance with better-organized sarcomeres, characterized by the presence of periodic Z-disks, H zones, and actin–myosin assemblies. Histological staining showed that TA muscle was severely damaged accompanied with fat deposition, inflammatory infiltration and fibrosis after hindlimb ischemia, while rhCol promoted regeneration with a large number of neo-myofibers and vascularization accompanied by reduced inflammation and fibrosis.

**Discussion and Conclusion:** In this study, we showed the protection of both rhCol I and rhCol III on structural and function of skeletal muscle after hindlimb ischemia through promoting neo-myofiber regeneration, vascularization and repressing inflammation, fibrosis. This rhCol treatment on ischemic skeletal muscle holds good translational potential in terms of scalability, reproducibility and clinical safety.

## A nanoworm-based gene delivery vehicle for stromal cell-based therapy

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Title: A nanoworm-based gene delivery vehicle for stromal cell-based therapy

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Category: SYIS (Student and Young Investigator Section)/ Design and Application of Biomaterials

**Introduction:** Mesenchymal stromal cells (MSCs) have promising therapeutic potential for disease treatment and tissue regeneration. When genetically engineered to express chemokine receptors and growth factors, MSCs can engender stronger homing ability to inflamed tissues and anti-inflammatory outcomes. Unfortunately, gene transfection of MSCs (or stromal cells in general) is challenging because of concerns over safety (when using viral vectors) and inadequate cellular uptake (when using non-viral transfection agents). A safe and efficient method of gene delivery into stromal cells is highly desirable for cell-based therapy.

**Methods:** We present a one-dimensional wormlike nanostructure (nanoworm, NW) for delivering therapeutic mRNA into MSCs. We evaluate the performance of this NW to facilitate gene expression in vitro and in vivo, and address if NW-enabled MSC-based therapy can alleviate tissue fibrosis.

**Results:** The NW can not only enter MSCs without the use of cationic and lipophilic transfection agents, but also escape from intracellular acidic compartments, thereby leading to the expression of the delivered mRNA without affecting the cell viability. By selecting the appropriate mRNA cargo, such genetically modified MSCs can efficiently home to the inflamed tissues upon systemic injection and alleviate inflammation.

**Discussion and conclusions:** Careful design of bionanomaterials can support efficient gene delivery into stem cells and endosomal escape. We envision that our NW delivery platform to be applicable to other types of stem cell-based therapies for tissue engineering and regeneration.

**Acknowledgements:** This work was supported General Research Fund (project no.: 14300221) by the Hong Kong Research Grants Council and Health@InnoHK scheme by the Innovation and Technology Commission.

## A Randomised Controlled Trial to Investigate Early Results of Pulsed Electromagnetic Field Therapy in Achilles Tendinopathy

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Introduction: Achilles tendinopathy is a debilitating clinical condition resulting from an altered Achilles tendon structure and mechanical properties. It impairs lower extremity function during activities of daily living and athletic capability. There are currently many limitations in the existing non-surgical treatment options, including exercises, orthotics, laser therapy, manual soft-tissue mobilization, extracorporeal shockwave therapy, and injection therapies (1); many patients requiring major surgery such as tendon transfer due to the poor functional improvement with conservative treatments.

Most individuals with Achilles tendinopathy suffer from significant pain, which detracts from their motivation to engage in eccentric exercise. Pulsed electromagnetic field therapy (PEMF) is a non-invasive treatment that effectively alleviates pain associated with musculoskeletal disorders (2). By incorporating PEMF into a treatment plan, patients may experience rapid pain relief and increased adherence to eccentric exercise. This could lead to a reduction in discomfort during these exercises, thereby promoting adherence to rehabilitation protocols.

Since Achilles tendinopathy remains challenging to manage successfully, this study aims to investigate the clinical effectiveness of PEMF as a treatment adjunct to eccentric exercise in patients with Achilles tendinopathy. The objective is to establish whether PEMF plus eccentric exercise in people with Achilles tendinopathy improves self-reported pain and function compared to eccentric exercise.

Subjects and Methods: This prospective, randomised, controlled study assessed the outcome of two different rehabilitation protocols in participants with Achilles tendinopathy. Participants were randomly allocated into the two treatment groups – active PEMF plus eccentric exercise group and sham PEMF plus exercise group. All participants completed a rehabilitation program: a progressive Achilles tendon-loading strengthening program for 12 weeks.

Participants in the intervention group will be exposed to PEMF treatment by a PEMF device (Quantum Tx, Singapore) (Figure 1). The active PEMF does not produce heat or cause any sensation to the tissue, which allows the participants to be blinded to the treatment. Participants in the control group will receive a sham exposure with the same PEMF device.

The primary outcome was the Victorian Institute of Sports Assessment – Achilles (VISA-A) questionnaire score, explicitly designed for Achilles tendinopathy. VISA-A was used to evaluate pain and symptom severity with activity in participants with AT. Chinese-speaking participants completed the validated Chinese VISA-A questionnaire (3). The VISA-A questionnaire covered three domains associated with AT: pain, function, and sporting activities. A deterioration in self-reported pain, function, and sporting activities was the critical reason patients with AT came for medical consultation. Improving questions covered in VISA-A was often the rehabilitation goal for these patients. The secondary outcome is the Short Form-36 questionnaires (SF-36) used to evaluate health-related quality of life.

Results: Sixty-four participants with Achilles tendinopathy were enrolled in a randomised controlled study. Regarding VISA-A scores, there was no significant difference in participant demographics between PEMF and sham groups. Both treatment groups showed significant improvements in the

VISA-A score at week 8. In the PEMF group, the VISA-A scores significantly improved from baseline ( $60.7 \pm 14.2$ ) to week 8 ( $70.7 \pm 16.8$ ) ( $p=0.0001$ ). In the Sham group, the VISA-A scores improved from baseline ( $55.4 \pm 16.8$ ) to week 8 ( $64.5 \pm 20.1$ ) ( $p=0.0001$ ). However, there was no significant difference between treatment groups at week 8.

The SF36 Physical Function domain was the most promising secondary outcome. In the PEMF group, the physical function scores improved significantly from baseline ( $69.2 \pm 19.0$ ) to week 8 ( $78.7 \pm 17.4$ ) ( $p=0.013$ ). In the Sham group, the physical function scores were improved from baseline ( $59.7 \pm 21.7$ ) to week 8 ( $69.3 \pm 24.6$ ) ( $p=0.064$ ). The SF36 Physical Function domain improved only in the PEMF group but not in the Sham group. No significant differences were found in other SF36 domains among the PEMF group.

**Discussion and Conclusion:** Achilles tendinopathy is a challenging clinical condition to address. Our randomized study on using PEMF as an adjunct therapy to eccentric exercise for this condition is the first of its kind. PEMF demonstrated encouraging symptomatic relief during the clinical trial and no adverse occurrences. While participants with Achilles tendinopathy who underwent eccentric exercise treatment did not experience more significant self-reported pain and improved function with PEMF therapies, further research studies could explore PEMF's carry-over effects on insertional and midportion Achilles tendinopathy.

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## 3D-printed NIR-responsive shape memory polyurethane/magnesium scaffolds with tight-contact for robust bone regeneration

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Title: 3D-printed NIR-responsive shape memory polyurethane/magnesium scaffolds with tight-contact for robust bone regeneration

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Category: Design and Application of Biomaterials

Background: Poor contact of grafts with defective bones and insufficient osteogenic activities lead to increased loose risks and unsatisfied repair efficacy. Although self-expanding scaffolds were developed to enhance bone integration, the limitations on the high transition temperature and the unsatisfied bioactivity hindered greatly their clinical application.

Methods: In this work, we report a near-infrared-responsive and tight-contacting scaffold that comprises of shape memory polyurethane (SMPU) as the thermal-responsive matrix and magnesium (Mg) as the photothermal and bioactive component. The SMPU/Mg scaffolds with different Mg contents were fabricated by low temperature rapid prototyping (LT-RP) 3D printing technology.

Results: The composite SMPU/Mg scaffolds possess a homogeneously porous structure. The mechanical properties of Mg containing scaffolds were significantly improved. The SMPU/Mg scaffolds exhibited stable photothermal effects, and very fast NIR-responsive shape memory property. The SMPU/4 wt%Mg scaffold had a shape fixity ratio (Rf) of ~93.6% and shape recovery ratio (Rr) of 95.4%. Cell studies demonstrated that the released Mg<sup>2+</sup> could promote the osteogenic differentiation of the BMSCs. The in vivo bone regenerative potential of the scaffolds was evaluated in a mouse calvarial defect model. The SMPU/4 wt%Mg scaffolds exhibited very tight contact with the defect after NIR triggered shape recovery, and finally achieved much more newly regenerated bone 12 weeks post implantation.

Discussion and Conclusion: The multiple functions of Mg particles have been explored for the first time to realize NIR triggered shape recovery of SMPU/Mg scaffold for tight contact and Mg<sup>2+</sup> release to promote osteogenesis. We envision this scaffold can be a clinically effective strategy for robust bone regeneration.

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## Anti-inflammatory strategies promote repair of osteochondral defect in osteoarthritis

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**Background:** Osteochondral defects (OCD) are common in osteoarthritis (OA) and remain an unresolved clinical problem due to the chronic inflammation in the joint. Despite the development and testing of numerous biomaterials for osteochondral defects, the inflammatory environment has often been ignored. In order to address this underlying pathophysiology, we utilized two anti-inflammatory strategies for fabricating osteochondral tissue engineering materials. One strategy involved the use of the anti-inflammatory small molecule cinnamaldehyde (CIN) from traditional Chinese medicine [1], which we incorporated into a biphasic porous and degradable scaffold. The other strategy involved the use of bone marrow-derived mesenchymal stem cell (BMSC)-laden 3D-bioprinted multilayer scaffolds. In this study, we aimed to evaluate the effects of these two scaffolds on promoting osteochondral regeneration in OA-OCD animal models.

**Methods:** The biphasic porous scaffolds (PK/PTC) were composed of poly lactic-co-glycolic acid with kartogenin for cartilage repair, and PLGA and  $\beta$ -calcium phosphate with CIN for subchondral bone repair. We evaluated the scaffolds using our well-established OA-OCD rabbit model. The effects of BMSC-laden scaffolds on osteochondral defect repair were investigated in a rat model of medial meniscectomy-induced OA.

**Results:** The biphasic scaffold PK/PTC promoted subchondral bone and cartilage regeneration, and reversed subchondral osteosclerosis caused by inflammation in the critically sized OA-OCD rabbit models after 16 weeks of implantation. BMSC-laden scaffolds facilitated chondrogenesis by promoting collagen II and suppressing interleukin 1 $\beta$  in osteochondral defects of the femoral trochlea after 12 weeks of implantation. Additionally, BMSC-laden scaffolds significantly improved joint function of the injured leg with respect to the ground support force, paw grip force, and walk gait parameters.

**Discussion and Conclusion:** These findings support the use of anti-inflammatory small molecules or BMSC-laden scaffolds to simultaneously inhibit joint inflammation and promote cartilage defect repair in OA joints.

**Acknowledgement:** This work was supported by the collaborative project from the National Key R&D Program of China and Innovation and Technology Fund Mainland-Hong Kong Joint Funding Scheme (Nos. 2021YFE0202300 and MHP/011/20).

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## Multiscale design of 3D hydrogel bioink with ROS scavenging and retina tissue regeneration

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**Background:** Retinal oxidative stress damage, associated with conditions such as glaucoma, macular degeneration, and diabetic retinopathy, poses a significant challenge for the development of targeted and efficacious therapeutic interventions.

**Methods:** In this study, we present the design and synthesis of a functional hydrogel, nitrocinnamic esterified gelatin (GelCA), which addresses this challenge through an innovative drug delivery system. GelCA is synthesized by grafting cinnamic acid onto natural gelatin polymers, facilitating photo-crosslinking and enhancing pi-pi stacking drug adsorption functionality. To augment the antioxidant properties of the hydrogel, we employed polydopamine nanoparticles (PDA NPs) as drug carriers, with the natural antioxidant curcumin (Cur) adsorbed onto their surfaces. This combination leverages the known antioxidant capabilities of both polydopamine and curcumin to create a more effective treatment option. The drug-laden nanoparticles were subsequently encapsulated within the GelCA hydrogel matrix, ensuring a controlled and localized release of the therapeutic agents.

**Results:** Experimental results demonstrated that the multifaceted Cur@PDA NPs adsorbed GelCA hydrogel exhibits excellent injectability, biocompatibility, and antioxidant capabilities. The innovative hydrogel design allows for effective drug delivery and targeted treatment, potentially leading to improved patient outcomes across various retinal disorders.

## Rapid Biofabrication Of Cell-free, Anisotropic Collagen Tissues

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**Introduction:** The posterior rectus sheath in the abdominal wall presents a distinct structural arrangement where the collagen fibres are oriented transversally to support the intra-abdominal pressure of the internal organs. These features of the native tissue are often overlooked in the design of meshes for the repair of hernias, where the mismatch of properties is thought to be one possible cause of failure of these implants. Various techniques have been developed to create anisotropic scaffolds, but they either rely on the ability of cells to re-arrange the matrix or use expensive and highly specialised equipment.

**Methods:** We developed a novel, inexpensive and rapid technique to produce acellular, aligned collagen sheets by combining horizontal shear flow (HSFlow) with the established RAFT method using rat tail collagen type I. We validated and characterised the method by measuring thickness and fluid loss compared to RAFT controls; the degree of alignment was also measured indirectly, by measuring alignment of human dermal fibroblasts (HDFs) cultured on the constructs, and directly, by quantifying collagen fibre alignment through Second Harmonic Generation microscopy (SHG).

**Results:** Measurements of fibril alignment revealed a significant difference overall between HSFlow and control samples, where both cells and collagen fibres showed alignment in the direction of shear flow, compared to the randomly aligned RAFT controls. SHG measurements confirmed these results showing a highly aligned structure of the HSFlow construct compared to the RAFT randomly aligned controls (Figure). Mechanical properties were also measured and revealed that HSFlow does not appear to improve the strength of the constructs despite the improved alignment, therefore further optimisation is needed to strengthen the constructs.

**Discussions and Conclusions:** We successfully developed a novel and rapid technique to generate flat sheets of aligned type I collagen without the contractile ability of cells to re-arrange the ECM. This method has potential to be used in the fabrication of a scaffold to mimic anisotropic tissues for regenerative medicine and particularly for biomimetic and biocompatible meshes for abdominal hernia repair.

## Novel supramolecular self-assembled nanocarrier systems for drug and gene delivery

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Title: Novel supramolecular self-assembled nanocarrier systems for drug and gene delivery

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Category: Design and Application of Biomaterials

Supramolecular host-guest chemistry has offered a powerful and convenient approach for fabricating complicated nanostructures self-assembled from individually tunable molecular building blocks. In the meantime, it has been a challenge to incorporate multiple functional features into a single drug and gene carrier system to overcome numerous hurdles during the delivery of drugs and genes. Usually, controlling molecular architectures and compositions of a multi-functional carrier system for optimizing delivery efficiency requires multi-step chemical synthesis and conjugation processes. Herein, we demonstrate a supramolecular approach for building multifunctional carrier systems with controllable molecular architectures based on the host-guest chemistry of cyclodextrins.

A system has been developed based on rationally designed host-guest complexation between a  $\beta$ -cyclodextrin-based cationic host polymer and a library of guest polymers with various PEG shape and size, and various density of ligands.

The host polymer is responsible to condense and load/unload siRNA, while the guest polymer is responsible to shield the vehicles from non-specific cellular uptake, to prolong the circulation time, and to actively target tumor cells. A series of siRNA vehicles with precisely controlled molecular architectures through a simple assembly process allow for a rapid optimization of siRNA delivery vehicles in vitro and in vivo for efficient targeted delivery of therapeutic siRNA-Bcl2 for tumor therapy. The good correlation between in vitro and in vivo data indicates this is a useful screening tool for targeted gene delivery vehicles.

We have developed a novel and powerful platform enabling precise control of molecular architectures and rapid optimization of gene delivery vehicles in vitro and in vivo for therapeutic gene delivery and targeted cancer therapy.

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## An injectable and biodegradable high-strength iron-bearing brushite cement for bone repair and vertebral augmentation applications

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**Background:** In clinic, autologous bone graft is still the gold standard for bone repairing, despite of its poor availability, donor site morbidity and long operation time. Calcium phosphate cement (CPC) such as brushite has been extensively investigated in the fields of orthopedics and dentistry. However, the main drawbacks of brushite cement lie in its inferior mechanical strength and poor injectability, which hinder its further applications in load-bearing conditions and minimally invasive surgery. The purpose of the study is to develop a novel brushite cement with good injectability and high mechanical strength.

**Methods:** In this study, an injectable brushite cement that contains monocalcium phosphate monohydrate (MCPM) and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) as its solid phase and ammonium ferric citrate (AFC) solution as the aqueous medium was designed to have high mechanical strength.

**Results:** The optimized formulation achieved a compressive strength of  $62.8 \pm 7.2$  MPa, which is above the previously reported values of hand-mixing brushite cements (Fig.1). The incorporation of AFC prolonged the setting times and greatly enhanced the injectability and degradation properties of the cements. In vitro and in vivo experiments demonstrated that the brushite cements exhibited good biocompatibility and bone regeneration capacity. The novel brushite cement is promising for bone healing in load-bearing applications.

**Discussion and Conclusion:** An injectable iron-bearing brushite cement with ultra-high mechanical strength was prepared in the presence of ammonium ferric citrate. The addition of ammonium ferric citrate prolonged the initial and final setting times, improved the mechanical properties and injectability, and accelerated the degradation of brushite cements. In vitro studies have demonstrated that the novel brushite cements exhibit good biocompatibility and can restore the mechanical function of impaired vertebra. In vivo experiments have shown that the brushite cements possess excellent bone repairing ability, indicating their great potential for load-bearing applications in the orthopedic field.

**Acknowledgement:** This work was supported by National Key R&D Program of China (2020YFC1107401), and National Natural Science Foundation of China (82002275, 81925027)

## Spatial Lipid Atlas of Human Cartilage Reveals Disease-specific Lipid Metabolic Signatures for Cartilage Regeneration

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Title: Spatial Lipid Atlas of Human Cartilage Reveals the Disease-specific Lipid Metabolic Signatures for Cartilage Regeneration

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Category: Design and Application of Biomaterials

### Background:

Lipid is a fundamental constituent of all living organisms. Ectopic accumulation of lipids is regarded as the biomarker for many diseases and aging. Aberrant cholesterol and fatty acid metabolisms were reported to induce osteoarthritis (OA), indicating the importance of maintaining cartilage lipid homeostasis. However, the detailed composition and distribution of lipids in human cartilage remain elusive. To investigate the heterogeneous lipid metabolism and identify therapeutic targets for cartilage regeneration ,

### Methods and Materials :

we established a high-resolution spatial lipid atlas of healthy and early OA human cartilage by integrating matrix-assisted laser desorption ionization mass spectrometry (MALDI/MS), lipid chromatography-mass spectrometry (LC/MS), and digital spatial profiling.

### Results:

LC/MS of human cartilage lipid extracts identified 564 lipids belonging to 5 lipid superclasses. By regionally deciphering the lipid contents across different areas, we identified 3 distinct lipid metabolic signatures, resulting in a layer-specific distribution of phospholipids and fatty acids in the upper and lower cartilage. We also discovered a disease-specific lipid signature, characterized by regional enrichment of lipid A, which is only distributed in the upper part of OA cartilages. Chondrocytes treated with lipidA showed increased ribosomal and mitochondrial activities and intra-articular injection of lipidA could help alleviate the cartilage loss in OA mice, indicating the accumulation of lipidA might be a rescue signal in the early-stage OA.

### Discussion and conclusion:

This study provided a panorama of lipids in human cartilage that uncovered the identity of the spatially distributed lipid and their corresponding functions in the different regions of cartilage, which not only provided lipidA as biomarkers and potential therapeutic target for early OA, but also proposed a novel strategy to achieve location-specific reconstruction and drug delivery of cartilage by incorporating the layer-specific lipids.

## Intrinsic differences between human mesenchymal stromal cells and iPSC-derived multipotent cells in generating cartilage tissue

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**INTRODUCTION:** Autologous chondrocyte implantation (ACI), the most frequently employed cell-based therapy clinically, is limited by low donor tissue availability, donor site morbidity and loss of chondrocytic phenotype during in vitro expansion. Therefore, other cell types, such as mesenchymal stromal cells (MSCs) derived from different sources, have been tested for their cartilage regenerative capacity (1). However, hypertrophic conversion often ensues, resulting in tissue ossification and apoptosis of the differentiated MSCs. Recently, multipotent progenitor cells (iMPCs) created from induced pluripotent stem cells (iPSCs) have been proposed as an alternative cell source for creating articular cartilage (2). In the studies conducted by us and other teams, bone morphogenetic proteins (BMPs) were shown to significantly enhance transforming growth factor- $\beta$  (TGF- $\beta$ )-induced iMPC chondrogenesis. In contrast, TGF- $\beta$  alone is sufficient to induce robust chondrogenesis of human primary mesenchymal stromal cells (MSCs). Currently, the mechanism underlying this difference between iMPCs and MSCs has not been fully understood. In this study, we first compared the potential of iMPCs and MSCs-derived cartilage in repairing chondral injury in rats, and then investigated the mechanistic difference to understand why iMPCs and MSCs behave differently in responding to TGF $\beta$ .

**METHODS:** With IRB approval, human bone marrow-derived MSCs were obtained from the femoral heads as previously described (3). Human iPSCs were reprogrammed from human bone marrow-derived MSCs. iPSCs were first induced into the multipotent cells (iMPCs) and then subjected to chondrogenic culture in pellet. 1) Repair of osteochondral defects in nude rats. This study was approved by IACUC. To examine the capacity of iMPCs for repairing cartilage defects in the knee joint, we implanted the iMPCs- or MSCs-derived cartilage pellets into surgically created osteochondral defects in the patellofemoral groove of nude rat knee joints. After 8 weeks, cartilage repair was assessed. 2) Phosphorylation of Smad 1/5 and Smad2/3 during the chondrogenesis of MSCs and iMPCs. Basic chondrogenic medium (BM, high-glucose DMEM supplemented with 1% antibiotic-antimycotic, 0.1  $\mu$ M dexamethasone, 40  $\mu$ g/mL L-proline, 10  $\mu$ g/mL ITS, 50  $\mu$ g/mL ascorbate 2-phosphate) were supplemented with different growth factors as indicated below: 100 ng/mL BMP-6 and/or 10 ng/mL TGF- $\beta$ 3. BMP pathway inhibitor LDN193189 (LDN) was introduced into iMPC culture at day 0, with the treatment lasting 7, 14 or 21 days. Western blot was used to assess levels of different proteins in different groups. 3) The expression level of ALK1/ALK5. Single cell analysis was utilized to determine the differential expression of both ALK1 and ALK5 among iMPCs. Western blot was used to assess levels of ALK1 and ALK5 at day0, and the differentiation stage(day 7, day14) for both BMSCs and iMPCs.

**RESULTS:** Compared to the BMSC, optimized chondrogenic medium (supplemented with TGF $\beta$ 3 and BMP6) led to robust cartilage formation with less hypertrophy for iMPCs in both in-vivo and in-vitro experiments. Interestingly, TGF $\beta$ 3 induced a higher level of P-Smad2/3 in iMPCs than in MSCs. Both BMP6 and TGF $\beta$ 3 induced the phosphorylation of Smad1/5 in MSCs, which was confirmed by the increased expression of inhibitor of DNA binding 1 (ID1). In contrast, TGF $\beta$  alone was not able to induce the phosphorylation of Smad1/5 in iMPCs. LDN treatment blocked the activation of Smad1/5 in iMPCs and GAG deposition, which clearly demonstrated that the activation of Smad1/5 is necessary for a successful chondrogenesis of iMPCs. Moreover, the TGF $\beta$  related surface receptors were detected, iMPCs expressed a significantly higher level of ALK 5 but a lower level of ALK 1 when compared to MSCs. Similar trends were also observed by testing MSCs from different patients and iPSCs from different sources. Currently, we are investigating whether the imbalanced expression of ALK1/ ALK5 could be the reason lead to the iMPC chondrogenic behavior.

**DISCUSSION:** In this study, we showed the combination of TGF $\beta$ 3 and BMP6 resulted in robust hyaline-like cartilage formation in both in vitro and in vivo studies for iPSCs, which overcomes the limitation with using MSCs for cartilage repair. In the mechanistic study, we revealed the critical role of Smad1/5 in initiating chondrogenesis of both MSCs and iPSCs, and further elucidated the mechanism may attribute the chondrogenic behavior of iPSCs.

**SIGNIFICANCE:** We demonstrated the superior potential of iPSCs in generating hyaline cartilage in both in vitro and in vivo studies. In addition, we conducted mechanistic study to understand why MSCs and iPSCs respond differently to chondro-induction factors.

**REFERENCE:** 1M.F. Pittenger et al. NPJ Regen Med, 2019, 2N. Nakayama et al. Cell Mol Life Sci, 2020, 3Xiang et al. Clin Transl Med, 2022



## Engineered hierarchical microdevices enable pre-programmed controlled release for postsurgical and unresectable cancer treatment

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Title: Engineered hierarchical microdevices enable pre-programmed controlled release for postsurgical and unresectable cancer treatment

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Category: Design and Application of Biomaterials

Background: Drug treatment is required for both resectable and unresectable cancers to strive for any meaningful patient outcomes improvement. However, the clinical benefit of receiving conventional systemic administrations is often less than satisfactory. Drug delivery systems are preferable substitutes but still fail to meet diverse clinical demands due to the difficulty in programming drug release profiles.

Methods: In this work, we introduce a microfabrication concept, termed Hierarchical Multiple Polymers Immobilization (HMPI), and engineer biodegradable polymers-based hierarchical microdevices (HMDs) that can pre-program any desired controlled release profiles. Based on the first-line medication of pancreatic and breast cancer, controlled release of single gemcitabine and the doxorubicin/paclitaxel combination in situ for multiple courses was implemented, respectively. Preclinical models of postsurgical pancreatic, postsurgical breast, and unresectable breast cancer were established, and the designed hierarchical microdevices demonstrated well-tolerable and effective treatments for inhibiting tumor growth, recurrence, and metastasis.

Results and Discussion: The hierarchical microstructures can be tailored to pre-program the drug release profiles. The clinical regimens-specified PPCR of single-drug (GEM) and multi-drug (DOX/PTX) in situ for multiple courses was presented by elaborately designing the HMDs, and their efficacy and safety were demonstrated in preclinical pancreatic and breast cancer models, respectively. The HMPI strategy thus offers the possibility of customizing and optimizing controlled drug release through pre-programmed design and precise microfabrication, representing potential alternatives to dealing with postsurgical and unresectable tumors.

Conclusion: The proposed HMPI strategy allows us to create tailorable and high-resolution hierarchical microstructures for pre-programming controlled release according to clinical medication schedules, which may provide promising alternative treatments for postsurgical and unresectable tumor control.

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## Self-assembling of Anisotropic Nanoclay Gels for Drug Delivery

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**Introduction:** Emulating the three-dimensional (3D) organisation of biochemical cues present in the native cellular microenvironment is likely to be key to generate biomaterials with distinct levels of functionality. However, despite advances in tissue engineering (TE), building structures incorporating stable 3D-micropatterning of biochemical cues and that preserve their resolution with an increase in size has proven challenging<sup>1</sup>. Nanoclay-gels have established potential in TE due to their capacity to adsorb, retain and localise proteins bioactivity<sup>2</sup>. In this study, we report a unique property of clay nanoparticles to self-organise via reaction-diffusion process that supports localised delivery of 3D protein gradients with enhanced bioactivity for bone regeneration.

**Methods:** Suspensions of a synthetic smectite clay (Laponite<sup>®</sup>), were exposed to a solution containing biomolecules and ions present in blood plasma to support the self-assembly of 3D micro-patterned gels through a reaction-diffusion process. To characterise the phenomenon, the assembled structures were tested for their ability to pattern fluorescently labelled model proteins under different conditions known to affect diffusion process, including pH, ionic strength, incubation time, protein concentration and protein physicochemical properties. The structures were analysed using a range of imaging techniques, including brightfield, fluorescent, polarised light and electron microscopy. The bioactivity and safety of a structure containing a BMP 2 gradient was assessed in a 60-day murine subcutaneous implantation assay.

**Results:** Nanoclay/protein gel develops an internal degree of order that allows templating punctuated or gradual 3D gradients of all proteins. By changing parameters known to affect diffusion rate at the assembly step, such as concentration, ionic strength, incubation time and temperature, it was possible to demonstrate control over the spatial localisation of the proteins (Fig 1). With this method is possible the assembly of structures at scale with a range of dimensions (0.2 - 10mm) and shapes (droplets, cylinders, strings) while preserving the resolution of protein patterning. The assembled structures displayed a radial birefringence under polarised light, indicating the presence of periodical arrangements of nanoparticles. Finally, the in vivo study revealed that punctuated localisation of BMP-2 within the scaffold provided the potential to control the spatio-temporal formation of mature bone.

**Discussion & conclusions:** We demonstrated for the first time the potential to harness interactions between clay-nanoparticles and biomolecules present in physiological fluids to design scaffolds with complex biochemical gradients, dimensions, and shapes for bone with clinical relevance.

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## The Engineered Brain-like Constructs fabrication and its application for Research Models in Neuroscience

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Category: Tissue Engineering and Regeneration

**Background:** Engineered brain-like constructs with functionality can be used as an ideal in vitro models for biological research and clinical treatment. The constructs with patterned neural cells based on biomimetic design are fabricated by 3D bioprinting, which is a prevalent bio manufacturing technique. Compared to 2D cultures models, 3D brain-like constructs provide complex microenvironment for neural cells which efficiently mimic natural conditions, and hence 3D brain-like constructs are ideal alternatives to both 2D cultures and animal models.

**Methods:** In this work, we developed a series of engineered brain-like constructs with neural stem cells and primary neural cells to investigate mechanism during development of network, mimic in vitro alternative epilepsy models, and fabricate retina regenerative tissue. Brain-like constructs are printed by our standard printing and culturing process. Relevant valuation experiments are applied to these models to confirm their functionality in different research scenario.

**Results:** The engineered brain-like constructs are applied for development models of network, epileptic models, and retina regenerative tissue in our research. Implantation of brain constructs with neural stem cells in rats further demonstrates the great potentials in following tissue regeneration and medical treatment research. The elastic modulus is adjusted to around ~kPa, and the components of bioink are adjusted due to specific microenvironment. The constructs can be cultured more than 1 month and the viability of neural cells maintain above 80%. Drug testing and electrophysiological recording are obtained and relevant functionalities are demonstrated.

**Discussion and Conclusion:** A series of engineered brain constructs have been successfully developed to mimic functional brain tissue. A standard printing process for engineered brain-like constructs is proposed, and many types constructs with specific application scenario are developed by adjusting corresponding factors. Although the accuracy of cells patterning, especially single cell printing, is still required for further improvement, the fabricated constructs as defined 3D models with biological functionality will benefit future application in clinical treatment, regenerative medicine and new drugs research.

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## Biomimetic inorganic-organic hybrid nanoparticles from amorphous calcium phosphate clusters for biomedical applications

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Category: Design and Application of Biomaterials

Background: Amorphous calcium phosphate (ACP) plays an important role in the biomineralization of bone, teeth, and other tissues. Although ACP has good biocompatibility and versatile functions in vivo, its meta-stability and labile nature limit further biomedical applications. In this study, the biomineralization strategy was successfully utilized to develop ACP/PAA hybrid nanoparticles that exhibited efficient cellular degradation and may have good potential for the delivery of therapeutic molecules.

Methods: ACP and PAA (referred to as ACP/PAA) hybrid nanoparticles were synthesized using a biomineralization strategy. Different elements can be doped into ACP to give particular biological functions. Hybrid nanoparticles were encapsulated in GelMA microgels together for delivery of therapeutic molecules.

Results: ACP/PAA hybrid nanoparticles (~24.0 nm) were pH-responsive and could be efficiently digested under weak acidic conditions (pH 5.0-5.5). The internalization of assembled ACP/PAA nanoparticles by MC3T3-E1 cells occurred through endocytosis, indicated by laser scanning confocal microscopy and cryo-soft X-ray tomography. The assembled ACP/PAA particles could be digested in cell lysosomes within 24 h under weak acidic conditions, thereby indicating the potential to efficiently deliver encapsulated functional molecules. Our results demonstrated good biosafety of the inorganic-organic ACP/PAA hybrid nanoparticles, which can facilitate bone regeneration in vivo.

Discussion and Conclusion: Internalization of the Mg-ACP/PAA nanoparticles into MC3T3-E1 cells was observed through endocytosis, and lipid membranes retained intact without pore formation. We also found that the Mg-ACP/PAA nanoparticles were pH-sensitive and could be efficiently digested in lysosomes under weak acidic conditions. We observed an increase in intracellular Ca<sup>2+</sup> ion levels during nanoparticle digestion, which reached normal levels after incubation for 24 h, indicating good cytocompatibility. The addition of Zn<sup>2+</sup> ion showed significant effects on M2 polarization of monocytes. The in vivo experiment indicated that the ACP/PAA nanoparticles were nontoxic, which can obviously promote bone regeneration.

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## Cell-adaptable hydrogel promotes peripheral nerve repair by supporting endogenous cell infiltration and regulating macrophage polarization

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**Background:** Peripheral nerve injury (PNI) is a complex and challenging medical condition due to the limited ability of nerves to regenerate, resulting in the loss of both sensory and motor function. Hydrogels have emerged as a promising biomaterial for promoting peripheral nerve regeneration, while conventional hydrogels are generally unable to support endogenous cell infiltration due to limited network dynamics, thereby compromising the therapeutic outcomes.

**Methods:** The bisphosphonate (BP)-modified alginate (Alg) and glutathione (GSH) modified SF (SF-GSH) were synthesized using carbodiimide chemistry, and the Alg-Mg/SF hydrogels were cast by rapidly mixing the stock solutions of Alg-BP, SF-GSH, SF-MA, and Ca<sup>2+</sup>/Mg<sup>2+</sup>.

**Results:** Owing to highly dynamic interactions between bisphosphonate-modified alginate (Alg-BP) and Mg<sup>2+</sup>, the obtained hydrogels exhibited remarkable dynamic properties. The thiol-ene click reaction between GSH and MA groups on SF created the interpenetrating polymer network (IPN). To evaluate the performance of the cell-adaptable Alg-Mg/SF hydrogel, we implanted them into a 10-mm nerve defect in vivo. The adaptable hydrogels facilitated good cell-material interaction, including Schwann cell migration and neurite outgrowth, and this further contributed to muscle recovery from atrophy and nerve functional recovery within 8 weeks. We next investigated the mechanism underlying the enhanced nerve regeneration induced by Alg-Mg/SF hydrogels and found that such hydrogels could rapidly recruit macrophages in the short term and then promoted their M2 transition.

**Discussion and Conclusion:** These hydrogels have unique bioactivity and remarkable dynamic properties that promote SC infiltration and neurite ingrowth in vivo, leading to efficient muscle recovery from atrophy and nerve functional recovery. Moreover, the cell-adaptable Alg-Mg/SF hydrogel can rapidly recruit macrophages and then promote their M2 transition. Since both SCs and macrophages play critical roles in the regenerative microenvironment, this study demonstrates the potential of adaptable hydrogels as a promising approach for the reconstruction of microenvironment and the clinical treatment of PNI.

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## Fabricating a counter-gradient of multiple soluble factors by Multiphoton Microfabrication and Micropatterning (MMM) technology for reconstituting a complex soluble cell niche in vitro

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**Introduction:** In the native cell microenvironment, soluble factors (e.g., morphogens or growth factors) with different functions are presented to the targeting cells in a counter-gradient to dictate the cell fate toward different directions. For example, a counter-gradient of Activin A (ActA) and bone morphogenic protein 4 (BMP4) plays a key role in guiding the embryonic epiblasts toward forming endoderm and mesoderm/trophectoderm, respectively, in mouse gastrulation. However, the biomimetic in vitro model consisting of a spatially controlled counter-gradient of soluble factors for studying such biological events is limited. Multiphoton Microfabrication and Micropatterning (MMM) technology developed by our lab is a powerful platform that enables us to build a soluble cell niche on the protein substratum in a spatial- and quantity-controlled manner. The present work reports the feasibility of utilizing the MMM to fabricate a counter-gradient of two different soluble factors and its potential application in studying early development events such as recapitulating mouse gastrulation in vitro.

**Subjects and Methods:** A protein substratum was fabricated by two-photon initiated photo crosslinking of bovine serum albumin (BSA) in the presence of photosensitizer methylene blue (MB), followed by reacting with a hetero-bifunctional linker Sulfo-SANPAH. Then one type of soluble factor (e.g., epidermal growth factor (EGF)) was immobilized in a high-to-low concentration gradient on the Sulfo-SANPAH conjugated BSA substratum by the two-photon laser illumination using a high-to-low laser power gradient; and the other type of soluble factor (e.g., bone morphogenic protein 2 (BMP2)) was immobilized in a low-to-high concentration gradient in a similar way but using a low-to-high laser power gradient. The bioactivity of the BMP2 gradient was evaluated by immunofluorescence staining of nuclear pSmad 159 in mouse myoblast C2C12 cells cultured on such a gradient for 24 hours. The fabrication of a counter-gradient of ActA and BMP4 and the validation of its bioactivity and bio-functionality are underway.

**Results:** A counter-gradient of EGF and BMP2 can be fabricated on the surface of BSA substratum by changing the laser power in a cross-gradient manner. More importantly, the BMP2 gradient is bioactive in terms of triggering the differential nuclear translocation of pSmad 159, a hallmark of the activated canonical BMP signaling pathway, in C2C12 cells cultured on the gradient.

**Discussion and Conclusion:** The current work demonstrates the feasibility of the MMM technology in reconstituting a complex soluble cell niche consisting of a counter-gradient of two soluble factors with different functions. Further validation of bioactivity and functionality of the fabricated soluble cell niche with the counter-gradient of soluble factors is warranted, which will pave the way for its potential applications in studying early development events and answering other biological questions related to the processes controlled by multiple soluble factors in vitro.

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## Mg-containing implant modulates the characteristics of distinct mesenchymal progenitors to inhibit fracture callus fibrosis in long-term bisphosphonate-pretreated rats

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Title: Mg-containing implant modulates the characteristics of distinct mesenchymal progenitors to inhibit fracture callus fibrosis in long-term bisphosphonate-pretreated rats

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Category: SYIS (Student and Young Investigator Section) + Tissue Engineering and Regeneration

Background: Fracture callus fibrosis was found to be the key pathologic change in rats receiving long-term bisphosphonates (BPs) pre-treatment, which recapitulates the impaired fracture healing in atypical femoral fracture (AFF) patients with long-term BPs use clinically. Besides, dysfunction of specific mesenchymal progenitors has been demonstrated to play key roles in fibrosis-associated fractures, such as polytraumatic, radiation-associated, and diabetic fractures. Thus, the present study aims to investigate the anti-fibrotic effects of Mg-containing implants (MCI) in long-term BPs pretreatment-impaired femoral fracture healing in rats at single-cell resolution.

Methods: In this work, we used single-cell transcriptome sequencing (scRNA-seq) to depict the cellular atlas of fracture callus cells (FCCs) at 4 and 12 weeks post-fracture in Ctrl, BP, and BP-Mg groups respectively. The anti-fibrosis effects of Mg-containing implants and the validation of sequencing results were conducted in vivo and in vitro by performing immunofluorescence, flow cytometry, differentiation assay, real-time PCR, etc.

Results: We found that there were no significant differences in transcriptomes among Ctrl, BP, and BP-Mg groups at 4 weeks post-fracture, suggesting the relatively normal fracture healing process at the early stage in both BP and BP-Mg groups. However, as fracture healing progressed, the expression of fibrotic markers, such as Col1a1, Col3a1, Fn1, Acta2, and Tgfb1, was dramatically upregulated in BPs-treated rats while decreased by implantation of MCI. At the cellular level, two subsets of mesenchymal progenitors were defined, one was Grem1+ CD105+ CD90+, and another was Prx1+ CD90+. Interestingly, Grem1+ mesenchymal progenitors were dramatically increased in the BP-Mg group, accompanied by activation of the chemokine signaling pathway. In vitro experiments demonstrated that Prx1+ FCCs possessed greater myofibrogenic differentiation potential, while Grem1+ FCCs possessed higher osteogenic differentiation potential. Furthermore, BPs pre-treatment augmented the myofibrogenic potential of both Grem1+ and Prx1+ FCCs, while reducing the osteogenic potential of Grem1+ mesenchymal progenitors. By comparison, the implantation of MCI alleviated the pro-fibrotic effects of BPs on both Grem1+ and Prx1+ FCCs, while rescuing the attenuated osteogenic potential of Grem1+ FCCs obtained from BPs-treated rats.

Discussion and Conclusion: We demonstrated that MCI inhibited fracture callus fibrosis in long-term BPs-pretreated rats via differential modulation of Grem1+ and Prx1+ mesenchymal progenitors for the first time. Our study will shed new light on the potential development and application of Mg-containing devices in challenging musculoskeletal disorders associated with aberrant fibrosis.

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## Discovery of Muscle-Tendon Progenitor Subpopulation in Human Myotendinous Junction at Single-Cell Resolution

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- Title

Discovery of Muscle-Tendon Progenitor Subpopulation in Human Myotendinous Junction at Single-Cell Resolution

- Introduction

The myotendinous junction (MTJ) is a complex and special anatomical area that connects muscles and tendons, and it is also the key to repairing tendons. Nevertheless, the anatomical structure and connection structure of MTJ, the cluster and distribution of cells, and which cells are involved in repairing the tissue are still unclear.

- Subjects and Methods

We used single-cell RNA sequencing to analyze the distribution and function of cell clusters at the human MTJ, and validated the newly identified key regeneration cluster, MTP, and its related signaling pathway using in vitro and in vivo experiments.

- Results

We used bioinformatics analysis methods such as Seurat, Monocle, and StemID at the single-cell level to identify four major clusters of MTJ tissue, including stem cell, muscle, and the key regeneration cluster --muscle-tendon progenitor cells(MTP). We identified that this cluster simultaneously expresses muscle and tendon marker genes, and deduced that this cluster has the potential for bidirectional differentiation. Then, using histological staining and immunofluorescence staining techniques, we determined the location and morphological structure of MTP cluster - located at the junction of muscle and tendon cluster, distributed in a transparent red wine cup shape. Subsequently, by constructing an MTJ injury model, it was demonstrated that MTP has strong muscle and tendon regeneration ability. Finally, we also demonstrated the importance of the mTOR signaling pathway in maintaining MTP through in vitro addition of rapamycin and in vivo validation using mTOR-ko mice.

- Discussion and Conclusion

Our research conducted a comprehensive analysis of the heterogeneity of myotendinous junction, discovered a special cluster called MTP, provided new insights into the biological significance of myotendinous junction, and laid the foundation for future research on myotendinous junction regeneration and restoration.

## The circUbqln1 interacts with 14-3-3 $\zeta$ to inhibit collagen synthesis and promote osteoarthritis by regulating proline metabolism

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Osteoarthritis (OA) is an aging degenerative bone disease characterized by joint pain, stiffness, swelling and deformation. Unfortunately, clinical drug treatments for OA have shown limited effectiveness, and many patients eventually require surgical treatment to manage their symptoms. In-depth exploration of the pathogenesis and targets of OA will lay the foundation for treatment. In the present study, we found that cartilage-specific knockout XBP1 mice exhibited faster OA progression. Further studies confirmed that XBP1s can inhibit the expression of circUbqln1, and circUbqln1 can promote cartilage catabolism and inhibit anabolism. Mechanism studies have found that circUbqln1 can translocate to the nucleus with the assistance of phosphorylated 14-3-3 $\zeta$ , upregulate the transcriptional activity of the proline dehydrogenase (Prodh) promoter and PRODH enzyme activity, thereby promoting proline degradation and inhibiting collagen synthesis, and eventually leads to cartilage damage. All the data showed that circUbqln1 plays an important role in the occurrence and development of OA, and inhibiting the generation of circUbqln1 can effectively alleviate the cartilage damage of OA. This study provides new targets and new strategies for the development of OA therapeutic drugs.

## Mechanically conditioned multilayered angle-ply collagen scaffolds promote annulus fibrosus regeneration

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**Background:** Annulus fibrosus (AF) injury, as a result of intervertebral disc degeneration (IVDD) or herniation, is very difficult to heal due to its avascular characteristics. Tissue engineering strategies have become increasingly promising to treat IVDD. However, in light of the fact that AF displays an anisotropic microstructure that results in gradient mechanical properties, fabrication of tissue engineered AF remains challenging. In this study, we fabricated micropatterned collagen scaffolds with angle-ply structure mimicking the microstructural features of AF.

**Methods:** Cells were seeded on the micropatterned collagen membrane with a depth and width of 2  $\mu\text{m}$  and 10  $\mu\text{m}$  on the surface to detect the orientation and proliferation of cells on the collagen membrane. A customized loaded cell culture system was used to apply periodic tensile mechanical stimulation to the mono-layer aligned cell-collagen membrane, and then detect the gene expression of Col1a1, Col2a1 and Acan, and the protein expression of CAV1 and YAP. At last, BMSC sheets with cells and collagen fibers in alternating directions were rolled up together to form an angle-ply and multilayered tissue.

**Results:** Micro-patterns on the surface of collagen membrane direct BMSC alignment and ECM secretion. Cyclic mechanical loading further enhanced ECM production of BMSCs. Moreover, micro-patterning of the substrate and external mechanical loading have a positive synergistic effect on cell fate and ECM metabolism, which is beneficial for tissue repair and regeneration. Such changes of cell behavior and ECM section may be regulated by CAV1-YAP mechano-transduction system. In vivo study demonstrates the potential use of this biomimetic tissue for AF regeneration.

**Discussion and conclusion:** In the present study, we developed an angle-ply structure with multi-layer cell-collagen membranes to achieve AF regeneration. Such a strategy develops a reference and platform to investigate AF regeneration. Deeply exploring the biochemical composition of the native AF will provide theoretical support for the construction of tissue-engineered intervertebral disc structure.

**Acknowledgement:** This work was supported by National Natural Science Foundation of China (81925027, 31530024, 81672213, 32101103)

## Application of a 3D-printed hybrid fixation system to primary TKA in osteoporosis patients: A Multi-center, Randomized-controlled Trial

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**Introduction:** Osteoporosis is a critical risk factor for aseptic prosthetic loosening in total knee arthroplasty (TKA). Specifically, osteoporosis affects the integration between bone cement and tibial trabeculae in primary TKA. However, little is known the appropriate prosthesis for osteoporotic patients, and the ideal fixation in osteoporotic tibial is also lack and in debate. In this study, we developed a novel hybrid fixation strategy: combination of cementless 3D-printed porous sleeve in tibial stem and bone cement (tibial tray). The aim of this study is to compare the short-term effects of this novel hybrid fixation strategy with conventional cement fixation in osteoporotic patients.

**Methods:** We prospectively recruited 61 patients with osteoporosis (T score<-2.5) from October 2022 to February 2023. 33 patients received hybrid fixation in tibial (cementless 3D-printed porous sleeve and bone cement in tray), and 28 patients received the conventional bone cement fixation in tibial. Clinical results and outcome measures, radiographs, and complications were reviewed.

**Results:** Generally, no significant difference was found in terms of clinical results including joint function and pain score between hybrid and conventional fixation group at a mean 3 months follow-up. However, the hybrid fixation group demonstrated a significantly lower prosthetic movement ( $0.40\pm 0.16$  mm v.s.  $0.56\pm 0.06$  mm,  $p=0.028$ ).

**Conclusion**

The hybrid fixation strategy showed the same effect as conventional fixation prosthesis, and had a lower radiological prosthetic movement early after TKA, indicating a superior potential in preventing prosthetic subsidence. Long-term evaluation is required and the hybrid fixation could be an alternative for TKA in osteoporotic patients.

## Human iPS cell-derived astrocytes and oligodendrocytes for JC polyomavirus infection and replication

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Title: Human iPS cell-derived astrocytes and oligodendrocytes for JC polyomavirus infection and replication

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Category: Tissue Engineering and Regeneration

**Background:** JC polyomavirus (JCPyV) has been identified in patients with progressive multifocal leukoencephalopathy (PML) of the brain, in particular PML-type JCPyV infects astrocytes and oligodendrocytes in their brain. In contrast, the non-pathogenic (archetype) JCPyV persists in kidney tissue and around 70% of people worldwide are infected with archetype JCPyV. Since both archetype and PML-type of JCPyV replicate efficiently in COS-7 cell line, which carries the SV40 T antigen, COS-7 cells are generally used to study of JCPyV study. However, the presence of the SV40 T-antigen in COS-7 is also of concern in the development of anti-JC virus drugs and in the study of JC virus characteristics because the JC virus' own unique T antigen can be replaced by the highly active SV40T antigen, which promotes the replication of the virus. We have already established a culture system for PML-type JCPyV infection using human iPS cell-derived astrocytes (E. Shimbo, et al). In this study, following the astrocytes, we attempted to establish an infection system for JCPyV in oligodendrocytes.

**Methods:** Human iPS, 253G1, cells were differentiated through the neural stem cell stage into astrocytes or oligodendrocytes in the appropriate medium containing some growth factors, M1-IMRb strain was used as the JCPyV. The genomic DNA in the JCPyV particles in the culture medium was quantified by TaqMan real-time PCR. The production of viral particle and viral protein1 (VP1) was assessed by HA assay and immunostaining in astrocytes and oligodendrocytes, respectively.

**Results:** Human iPS cell-derived oligodendrocytes were confirmed by immunostaining for O4 and by oligodendrocyte-specific gene expression. Both human iPS cell-derived oligodendrocytes were infected with PML-type JCPyV and cultured for three weeks. Viral DNA, VP1 antigen, and HA were detected in both JCPyV-infected oligodendrocytes and astrocytes. Scanning electron microscopy also confirmed the extracellular appearance of the viral particles in the PML-type JCPyV-infected oligodendrocytes. In addition, like PML-type JCPyV, archetype JCPyV was found to infect and replicate in oligodendrocytes and astrocytes.

**Discussion and Conclusion:** We were able to establish infection and replication of PML-type JCPyV in astrocytes and oligodendrocytes derived from human iPS cells lacking the SV40 T antigen gene, which may be a useful system for analyzing JCPyV replication in the pathogenesis of PML. The finding that archetype JCPyV infects and replicates in oligodendrocytes and astrocytes suggests that the mutations in the JCPyV genome required for PML development may occur in the brain.

Reference: E. Shimbo, S. Nukuzuma, Y.I. Tagawa "Human iPS cell–derived astrocytes support efficient replication of progressive multifocal leukoencephalopathy-type JC polyomavirus." *Biochem. Biophys. Res. Commun.* 533:983-987. Doi: 10.1016/j.bbrc.2020.09.117. 2020

## Growth factors and topography direct multi-tissue regeneration of muscle-tendon units for rotator cuff repair

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Title: Growth factors and topography direct multi-tissue regeneration of muscle-tendon units for rotator cuff repair

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Introduction: Rotator cuff (RC) injuries are common but clinically challenging to treat, especially for large-to-massive tears that involve severe multi-tissue degeneration and poor healing. Although RC tears frequently occur at the bone-tendon interface, accompanying tendon retraction and subsequently the loss of mechanical load led to muscle atrophy, fat accumulation, and fibrosis formation, which can lead to poor patient outcomes. Despite tremendous advances, the successful repair of the torn tendon cannot reverse the prevent fatty degeneration (FD)-mediated muscle atrophy following massive RC tears. To address these limitations, we identified a growth factor (GF) cocktail comprised of fibroblast growth factor-2 (FGF-2), transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3), and insulin-like growth factor-1 (IGF-1), which can inhibit FD-mediated muscle atrophy and synergistically induced tenogenesis for muscle-tendon multi-tissue regeneration when combined with geometric cues.

METHODS: Our study aimed to establish a GF-based therapy that simultaneously induces tenogenesis and inhibits adipogenesis for regenerating tendon and rescuing FD, respectively. To induce tenogenesis, human mesenchymal stem cells (hMSCs) were cultured in serum-free media containing this GF cocktail and/or geometric cues. DNA staining using Hoechst 33342 dye as well as gene expression and immunostaining for Ki67 were performed to evaluate cell proliferation. Scanning electron microscopy (SEM) imaging and F-actin fluorescence staining were used to evaluate the cell morphological differences in response to topographical cues. Collagen secretion was assessed by Sirius Red staining and immunofluorescence staining was performed to detect tendon markers SCX and COL I. To study muscle FD, we developed in vitro and in vivo models that mimic FD using C2C12 myoblast cells, Fibro-adipogenic progenitors (FAPs) and rabbit subscapularis (SSC) muscle-tendon segmental injuries and applied our GF cocktail (FTI) to rescue FD following adipocyte formation. Cytochemical (Oil-Red-O) and immunofluorescence (MF-20, PPAR $\gamma$  and C/EBP $\alpha$ ) staining were performed to assess adipogenesis and myogenesis. TUNEL assay and 10x Genomics' single cell RNA-seq (scRNA-seq) were also performed to test whether apoptosis and/or transdifferentiation were responsible for the disappearance of adipocytes observed after GFs treatment in our in vitro model. Histological staining was performed to assess muscle FD in in vivo model.

RESULTS: To determine if our GF cocktail (FTI) was capable of promoting multi-tissue RC regeneration, i.e., enhancing tenogenesis and inhibiting adipogenesis, serum-free tenogenic studies were performed in vitro whereas muscle FD studies were performed in vitro and in vivo. In tenogenic studies, our GF cocktail promoted hMSC proliferation and induced elongated morphology, highly

oriented cell alignment, and increased tendon-related markers expression including aligned ECM secretion in response to tendon-like topography under serum-free conditions. Notably, the synergistic effects between tendon-like topography and FTI supplementation enhanced nuclear Scx and Col I protein levels. In muscle FD studies, in vitro experiments showed significantly reduced lipid droplet accumulation due to the apoptosis in FTI-treated C2C12 cells and FAPs. Our in vivo experiments showed local delivery of our GF cocktail using an acellular dermal matrix (ADM) and fibrin gel reduced fatty accumulation and promoted muscle regeneration. Together, these results demonstrate multi-tissue RC regeneration via promotion of tenogenesis and inhibition of muscle adipogenesis.

**Discussion and Conclusion:** Our study identified an optimal GF cocktail for RC multi-tissue regeneration, which promoted tenogenesis in vitro and inhibited adipogenesis under simulated FD in vitro and in vivo. Application of a serum-free, GF-supplemented cocktail supported robust cell proliferation and differentiation, which was synergistically enhanced in the presence of tendon-like topography. These findings can facilitate the generation of clinically relevant (large) numbers of tenocytes for cell-based therapy and inform future development of biomaterials for tendon tissue engineering. In addition, the C2C12 and FAPs in vitro models and rabbit SSC transection in vivo model showed that this tenogenic GF cocktail was useful in rescuing muscle FD, potentially due to transdifferentiation and apoptosis. A limitation here is the lack of muscle strength assessment, which will be addressed in future work. Together, our work paves the way for RC multi-tissue regeneration.

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## An injectable microporous scaffold assembled from bone-mimicking building blocks for bone regeneration

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**Introduction:** Injectable hydrogel scaffolds are promising in bone regeneration because they can be delivered by minimally invasive surgical method. However, their nano-porous nature limits nutrition transport, cellular infiltration, tissue ingrowth as well as vessel invasion, resulting in unsatisfied regenerative efficacy. Therefore, designing an injectable and microporous scaffold with enhanced cellular infiltration is favorable.

**Subjects and Methods:** In this work, we generated bone mimicking microgels and assembled them into a 3D macroscopic scaffold. We tested the injectability, microporosity, structural stability and mass transport ability of the scaffold. We also used a subcutaneous implantation model and a cranial defect model to evaluate the biological performance of the scaffold in vivo.

**Results:** The scaffold exhibited good injectability, interconnected microporosity and mechanical stability. Upon subcutaneous implantation, the scaffold promoted mass transport and enhanced cellular infiltration, new tissue ingrowth as well as vessel invasion. Furthermore, the scaffold was confirmed to regulate macrophage M2 polarization and stimulate macrophages to secrete beneficial cytokines such as IL10 and BMP2. These cytokines created an instructive immune microenvironment and could simultaneously recruit autologous stem cells and promote osteogenic differentiation. The in vivo results demonstrated that the scaffold achieved effective bone repair in calvarial defects of rats.

**Discussion and Conclusion:** We developed an injectable and microporous scaffold for bone regeneration. We demonstrated that the scaffold could promote new tissue development within the scaffold. In addition, the scaffold could create a beneficial osteo-immune microenvironment by inducing macrophages toward pro-regenerative M2 polarization. This work provides a promising candidate material for regeneration of bone defects.

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## Biodegradable zinc metal for orthopedic applications

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Traditional inert materials used in orthopedic implants have been associated with a multitude of complications, often necessitating secondary surgical interventions for removal. However, the advent of biodegradable materials, including magnesium (Mg), iron (Fe), and zinc (Zn) based alloys, has paved the way for a new approach to address these challenges. Over the past decade, zinc-based alloys have garnered considerable attention from researchers and have witnessed remarkable advancements. Boasting mechanical properties comparable to those of conventional metallic materials like titanium (Ti), zinc alloys offer a moderate degradation rate, rendering them highly promising as potential materials for applications such as internal fixation and bone augmentation, particularly in weight-bearing regions.

Under the guidance of Professor Yufeng Zheng and Professor Kerong Dai, our research team has undertaken the development of a range of zinc-based materials exhibiting biologically active and biocompatible characteristics. Through meticulous *in vitro* and *in vivo* investigations, we have endeavored to unravel the mechanisms underlying their biodegradability, mechanical performance, biocompatibility, and biological functionality, ultimately aiming to advance towards their clinical implementation in orthopedic surgery.

Our research efforts have yielded several zinc-based binary alloys, including Zn-Li, Zn-Sr, Zn-Mg, Zn-Cu, and Zn-Ag, as well as zinc-based ternary alloys, such as Zn-Li-Mn, Zn-Li-Ag, Zn-Li-Ca, and Zn-Li-Sr. Impressively, these materials have demonstrated remarkable efficacy in various orthopedic domains, including internal fixation, bone regeneration, and infection resistance. Nevertheless, our investigations have underscored the need for meticulous consideration of device design, the immune microenvironment at the implantation site, and the prevailing mechanical conditions when implanting zinc alloys.

By comprehensively reviewing the progress achieved in our research endeavors pertaining to zinc-based biodegradable materials, this report aims to not only shed light on our team's contributions but also identify the challenges encountered and present viable solutions. We trust that this review will serve as an invaluable reference for future translational investigations aimed at clinically translating zinc-based biodegradable materials for orthopedic applications.

### 3D cancer models: Challenges and opportunities for rare tumors

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#### Introduction

Cancer tissue engineering is an emerging multidisciplinary field aimed at growing cancerous cells onto porous biomaterial-based scaffolds and proper stimuli to ultimately reproduce three-dimensional (3D) tumor tissue-like constructs in vitro. These tissue models can reproduce cancer lesions very similar to those present in native tumor and can be viable for many weeks, making it possible to study cancer biology and assess therapies in a better reliable way [1]. Biomaterial scaffolds provide cells with a 3D structure that ultimately acts as a preliminary extracellular matrix (ECM), enabling mechanical, chemical and topographical features that can accomplish the characteristics of a specific tissue.

Rare cancers (i.e., incidence < 6 cases out of 100,000 in the European population per year) are estimated to account for about 20%-25% of all cancers and can affect all the sites of the body, with all solid rare cancers accounting for about 15%. As such, rare tumors are a very heterogeneous group of diseases, sharing similar problems: uncertainty of diagnosis, lack of therapies, poor research opportunities, difficulties in clinical trials, lack of expertise and of reference centers. In rare cancers, the onset of tumors arises since childhood, due to some hereditary genetic mutations, or will come with age when some genetic variants in the population that predispose to tumor development have accumulated other pathological mutations in the cells.

Among them, sarcomas (e.g. osteosarcoma, occurring in young patients), and rare head and neck cancers (e.g., sinonasal cancers) represent two types of tumors with low survival rate and poorly effective therapeutic options, which, due to the diverse nature of their ECM and tumor microenvironment (TME), offer interesting case studies for cancer tissue engineering approaches. Sinonasal cancers account for 3% of all cancers of head and neck district, with prevalence in some geographical areas [e.g., China, Japan, Italy (Tuscany and Eastern Piedmont)], and are associated to specific job categories, like carpentry and tannery.

#### Materials & Methods

We developed 3D models for osteosarcoma and sinonasal cancers, where cancer cell biology and interaction with the TME could be studied. To offer a scaffold platform for 3 rare cancers, i.e., squamous epithelium (squamous cell carcinoma; SCC), mucous epithelium (intestinal-type adenocarcinoma; ITAC and mucous melanoma; MM) and bone (osteosarcoma), 3 different scaffold types were set-up, based on polyhydroxybutyrate (PHB) and its copolymer poly(hydroxybutyrate-hydroxyvalerate) (PHBHV) [2]. By studying quaternary polymer-solvent systems, we designed collagen peptide/PHBV (50/50 w/w%) and chitosan/PHBV (5/95 w/w%) electrospun scaffolds for epithelial tissues, and nano-BaTiO<sub>3</sub>/PHB (5/95 w/w%) 3D printed scaffold for bony tissue. In addition, polyvinyl alcohol (PVA) was investigated due to its higher hydrophilicity with respect to polyhydroxyalkanoates and its ability to be produced as sponges with very suitable characteristics for nasal tissues [3]. PVA was blended with gelatin (G) under emulsion and freeze-drying at PVA/G 90/10 w/w% and 70/30 w/w% weight ratios. The pre-generation of bone ECM on 3D printed scaffolds was studied to evaluate the response of osteosarcoma cells to microenvironmental stimuli. We cultured tumor cell lines representative of the selected tumors, cell lines isolated from sinonasal tumor specimens, and their normal counterparts and characterized the obtained 3D models according to cell viability and morphology. The cancer/scaffold constructs were ultimately treated using innovative molecules targeting glucose metabolism synthesized in Pisa University and their antiproliferative and pro-apoptotic activity was assessed to demonstrate specific susceptibility of these rare cancers to these molecules.

#### Results & Discussion

The scaffolds were sterilized using absolute ethanol overnight. Commercial cancer cell lines (Saos-2 for osteosarcoma, FaDu for epithelial tissue tumor, and Caco-2 for mucosal epithelial tissue tumor, the latter to mimic ITAC) representative of the selected tumors and their normal counterparts (human mesenchymal stem cells for bone and HaCaT keratinocytes for epithelial tissues) were cultured on PHB-based scaffolds and the obtained 3D models characterized according to cell viability and morphology via metabolic tests and fluorescence microscopy.

Ten cell lines derived from patients affected by sinonasal tumors were isolated in Pisa hospital, including ITAC and MM, which were tested in our scaffold platform, demonstrating that the morphologic and genetic features of those sinonasal tumors could be mimicked in vitro. The results analyzed via histology and metabolic activity tests (AlamarBlue assay), showed that these sponges can replicate the main morphologic features of ITAC and MM sinonasal tumors. Among all, PVA/G 90/10 appeared to be a promising candidate for modeling ITAC.

Finally, we demonstrated the relevance of the TME by adding specific biomolecules, i.e., collagen peptides, polysaccharide (chitosan), and bone ECM. The effect of glucose inhibition drugs on ITAC 3D model is also elucidating new routes for treating this malignancy.

#### Conclusion

Studying the interactions between cancer cells and specifically patient-derived cancer cells with engineered scaffolds, allows in vitro 3D tumours useful for improving the understanding on carcinogenesis mechanisms to be made available and thus personalized therapies to be established. Our investigation offered a variegated set of 3D models for neoplasms of hard and soft tissues, useful for rare tumors. In our study, we demonstrated the exceptional processability of PHBHV into ultrafine fibres (useful to mimic the extracellular matrix of connective tissues, thus soft cancers) if blended with a biopolymer (i.e., collagen peptides or chitosan), and PHB in 3D printed filament structures, useful to mimic hard tissue cancers using nanoceramic as fillers. Finally, nasal mucosal tissues can be mimicked by other more hydrophilic scaffold types, such as PVA/G sponges.

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## Single-cell and spatial analysis reveal the interaction of CXCL5+ macrophages and POSTN+ fibroblasts in tendinopathy

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Title: Single-cell and spatial analysis reveal the interaction of CXCL5+ macrophages and POSTN+ fibroblasts in tendinopathy

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Category: Stem Cells and Cell-Based Therapies

### Introduction:

Tendinopathy is characterized by fibrotic scar tissue with decreased mechanical function and a high re-rupture rate, accounting for about 30% of musculoskeletal disorders and imposing a heavy social and economic burden. However, the cellular and molecular mechanisms underlying the pathogenesis of tendinopathy remain less understood.

### Methods:

Freshly prepared cell suspensions from four healthy and six tendinopathy human samples were performed immediately according to the manufacturer's protocol of 10 X Chromium 3' v3 kit. Spatial Transcriptomics (ST) slides were printed with four capture areas from two normal and two tendinopathy samples. The capture of gene expression information for ST slides was performed by the Visium Spatial platform of 10x Genomics. The generated data were analyzed with the Seurat package in R.

### Results:

In this study, we profile 75,428 cells from normal and pathological tendons to characterize the cellular composition and elucidate the microenvironmental regulatory mechanisms of tendinopathy. We identify the fibrosis-associated CXCL5+ macrophages and POSTN+ fibroblast subpopulations, which both expand in the fibrotic tendon. We further demonstrate that these two cell subsets are spatially adjacent in the pathological tendon microenvironment and mainly interact through the TNF- $\alpha$  signaling pathway, as demonstrated by spatial transcriptomics and immuno-fluorescent staining. Moreover, inhibiting TNF- $\alpha$  in vivo can significantly suppress the formation of fibrotic scars and mitigate the progression of tendinopathy.

**Discussion and Conclusion:** Our study has unveiled the detailed landscape of the pathogenesis microenvironment of tendinopathy at the in situ single-cell level and highlighted the potential value of identifying and developing therapeutic strategies targeting CXCL5+ macrophages, POSTN+ fibroblast, or the molecules involved in their crosstalk to improve tendinopathy treatment.

### Acknowledgment:

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## Research and Development of a Novel Biodegradable Mg Interference Screw for ACL Reconstruction

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**Introduction:** Magnesium (Mg) ions exhibit stimuli on release of pro-osteogenic neurotransmitters and pro-angiogenic factors (e.g. PDGF-BB, etc.), favoring bone regeneration and angiogenesis at the tendon graft integration towards the bone. As the activities of osteoclasts and osteoblasts are regulated by Mg ions, the re-innervation at the tendon-bone interface (TBI) may be enhanced in the presence of osteoblast- or osteoclast-derived slit guidance ligand 3 (SLIT3). Herein, we proposed the hypothesis that our developed novel Mg interference screws may promote the TBI healing through enhancement of type H vessel formation and re-innervation via modulation of activities of osteoblasts and osteoclasts.

**Subjects and Methods:** First, as the poor torque of currently designed screws is a challenging issue for the clinical use, the structure of the Mg interference screws was designed and optimized using finite element analysis (FEA) and experimental validation. Second, the novel Mg interference screws were tested in rabbit and goat models with anterior cruciate ligament (ACL) reconstruction through gait analysis, biomechanical testing, radiographic measurement, and histological assessment. The PLA and PEEK interference screws were used for rabbits and goats, respectively. After surgery, the knee samples were harvested at 6, 12, and 24 weeks post-surgery (n=4/time point/group for histology analysis while n=6/time point/group for gait, radiographic and biomechanical testing). Finally, a murine model with ACL reconstruction was performed to study the effects of Mg ions on angiogenesis (type H vessels) and re-innervation at the TBI via investigation of sensory-parasympathetic neural circuit. One-way ANOVA with Tukey's post-hoc correction was used for multiple comparison of data in more than two groups (p<0.05 set as significant difference).

**Results:** Both FEA and experimental validation data have confirmed the superior torque performance of the Mg interference screws with novel design (actual torque: 2.12 Nm vs. 6.08 Nm, p<0.01). In both rabbit and goat models, the load to failure of the femur-tendon graft-tibia complex during the tensile testing in the Mg group was significantly higher than that in the control group at 24 weeks post-surgery (rabbit: 62 N vs. 43 N, p<0.05; goat: 410 N vs. 320 N, p<0.05). Additionally, the dynamic laxity displacement was significantly reduced in the Mg group relative to the control group in both rabbit and goat models (rabbit: 0.25 mm vs. 0.36 mm, p<0.05; goat: 1.05 mm vs. 1.83 mm, p<0.05). Of note, the peri-tunnel trabecular bone thickness (Tb. Th) in the Mg group was significantly higher than that in the control group (rabbit: 0.18 mm vs. 0.05 mm, p<0.001; goat: 0.65 mm vs. 0.47 mm, p<0.001). In the murine model, compared to the control group, the Mg ion treated group showed profoundly increased type H vessel formation and re-innervation at the TBI while the block of the Mg ion channels (MagT1) abolished the effects.

**Discussion and Conclusion:** The biodegradable Mg interference screw may be a next generation of the tendon graft fixators favoring the graft osteointegration through modulation of type H vessel formation and re-innervation at the TBI.

## Electrical stimulation promotes the vascularization and functionalization of an engineered biomimetic human cardiac tissue

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### Introduction

The formation of multiscale vascular networks is essential for the in vitro construction of large-scale biomimetic cardiac tissues/organs[1]. Although a variety of bioprinting processes have been developed to achieve the construction of mesoscale and large-scale blood vessels, the formation of microvascular networks still mainly depends on the self-assembly behavior of endothelial cells(EC), which is inefficient and demanding without appropriate stimulus. To address this problem, We seek to promote the elongation and connection of endothelial cells in engineered cardiac tissue (ECT) by electrical stimulation (ES) to achieve vascularization.

### Subjectss and Methods

Bioinks composed of GelMA, fibrinogen, iPSC-CMs, and HUVECs were used for the bioprinting of the engineered cardiac tissue (ECT). The rheological properties, mechanical properties and biocompatibility of the GelMA-Fibrinogen hydrogel were tested. The ECTs were trained in a self-developed bioreactor according to a frequency enhanced stimulation plan(2Hz-5Hz) from day7 to day 17. Then we characterized the morphological changes of HUVECs through immunofluorescence and analyzed their gene expression. We also investigate the mechanism that drives HUVEC changes in ECT through qRT-PCR analysis. Finally, we analyzed the effects of electrical stimulation and endothelial cells on myocardialcell morphology, calcium treatment and contractile functions.

### Results

Between 35 °C and 15 °C, the addition of 0.5% or 1% fibrinogen slightly increased the viscosity of the material, but there was no obvious difference between the three below 15 °C. The GelMA-Fibrinogen hydrogel still maintained a good mechanical and printing properties. Moreover, the addition of fibrinogen can promote the proliferation of ECs. Compared with the non-stimulated group, ECs in stimulated groups became denser and more elongated on day 17. HUVECs also show hollow structures similar to capillary lumens. Electrical stimulation significantly increased the number of junctions and total branching length of the endothelial network by about 4 times. The expression of several endothelial transcription factors, including CD31, CD144, and CD34, were both upregulated, showing the initial phenomenon of vasculogenesis, such as proliferation, migration, and cell-cell interactions, occurred in endothelial cells in the ECT after electrical stimulation. The results also show that electrical stimulation does enhance the gene expression of ECs vascularization signal pathway (VEGFA-VEGFR), and enhances the secretion of angiogenic factors in cardiomyocytes, such as FGF2 and ANGPT1. Cardiomyocytes in stimulated groups formed ordered sarcomeres on day 24, and showed stable spontaneous contractility and similar calcium transient curves, with contractile frequencies of about 1.2Hz. The electrical stimulation increased the contraction strength of ECTs significantly, and what's more, cardiac tissue containing endothelial cells showed greater and uniform contractile displacement under the same ES conditions.

### Discussion and Conclusion

Biochemical stimuli and mechanical cues have proved to affect angiogenesis and network morphology[2]. However, further studies focusing on the use of electrical stimulation for microvascular organization, especially in engineered tissue, are required. In this study, we demonstrate that EC spreading and connection in ECT can be promoted by electrical stimulation, providing a novel method for the micro-vascularization of ECT fabricated by 3D bioprinting. These results show the trend of endothelial cell vascularization and there seems to be no significant difference between physiological intensity (2.5 V/cm) and high-intensity (5.0 V/cm) electric field. The effect of myocardial cells on endothelial cell vascularization cannot be ignored. We have detected that CMs increased the secretion of angiogenic factors including VEGFA, FGF2, and Ang1 under electrical stimulation, which may play an important role in the vascularization of HUVECs through the

cross-talk of two types of cells. We found that although there was no significant difference in the frequency of spontaneous contraction between the CM(ES) and CM-EC(ES), the latter showed stronger and uniform contraction behavior. Then, we detected that ECs in CM-EC(ES) group increased the expression of NRG1 after the stimulation, which may contribute to the better contraction performance of iPSC-CMs. This work has significant application potential for the construction of vascularized cardiac chambers, allowing the fabrication of thicker walls to enhance the ability of cardiac chambers to pump fluid, which in turn allows better application of ECT for clinical transplantation. In future work, we will print the biomimetic cardiac structure with large-scale channels to better mimic the native heart and consider introducing electromechanical composite signals to elevate the training effect.

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## Scaffold with high curvature pores promotes segmental bone defect repair by regulating skeletal stem cells

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**Background:** The reconstruction of large segmental bone defects remains a worldwide clinical challenge, but available treatment options for achieving biomechanically adequate restoration are currently limited. A common tissue engineering strategy to address this challenge involves the utilization of skeletal stem cells (SSCs) with scaffolds to mimic mature bone tissue (1). During bone repair, mechanotransduction of SSCs plays a crucial role in mediating angiogenesis-osteogenesis coupling (2, 3). However, approaches to regulate SSCs' mechanotransduction for the repair of large bone defects have been lacking. In this study, we aimed to investigate whether mechanobiological optimization of pore curvature in 3D scaffolds could promote segmental bone defect repair by regulating skeletal stem cells.

**Methods:** In this work, we fabricated a mechanobiologically optimized 3D printed biphasic calcium phosphate scaffold (BCP scaffold). Two types of scaffold designs were used: octet truss and Kelvin cell. All animal procedures were approved by the Southern University of Science and Technology Animal Care and Use Committee. Experiments were performed on 16-week-old female C57BL6 mice (N=27) and Prx1Cre; RosatdTomato mice (N=9). Subcutaneous implantation was performed to test biological compatibility. Scaffolds were implanted into 2 mm unilateral femoral segmental defects with external fixation. Defects without scaffolds were used as controls. At 2, 4, and 8 weeks after surgery, femurs were imaged by micro-computed tomography ( $\mu$ -CT), SSCs and angiogenesis-osteogenesis coupling markers were measured by immunofluorescence staining, collagen fibers were imaged with second harmonic generation (SHG). Data are presented as mean  $\pm$  standard deviation and were statistically analyzed by 1- or 2-way ANOVA with Tukey's post-hoc comparison or Student's t-test ( $\alpha=0.05$ ).

**Results:** The 3D printed BCP scaffolds with octet truss architecture promoted the recruitment of endogenous SSCs by providing a favorable mechanical microenvironment. We fabricated mechanobiologically optimized scaffolds (Figure 1A). Octet truss had higher crack resistance and mechanical strength (Figure 1B,  $P < 0.01$ ). After 2 weeks of subcutaneous implantation, octet truss scaffold had the highest Osterix (OSX) and type H vessel volume. Octet truss also had a higher number of perivascular osteoblasts (OSX+) in proximity to type H vessels at the corner of the scaffolds (Figure 1C,  $P < 0.0001$ ). After 2 weeks in a segmental defect, octet truss group had more paired-related homeobox protein 1 (PRRX1) +, leptin Receptor (LepR)+, and Gli1+ cells within the defects. Furthermore, the orientation of collagen fibers was aligned with scaffold surface (Figure 1D).

**Discussion and Conclusion:** We demonstrated that high pore curvature alone such as those in acute angles could increase the number of SSCs and promote segmental bone defect repair. Our findings suggest a promising approach to designing mechanobiologically optimized scaffolds to regulate SSCs' mechanotransduction to promote bone regeneration.

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## Primary Repair for Femoral-Side Anterior Cruciate Ligament Tears: A Preclinical Murine Model

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**INTRODUCTION:** The anterior cruciate ligament (ACL) is one common knee injuries. Compared to the limited effect of conservative treatment, the surgical intervention aims to restore knee stability and reduce the development of post-traumatic osteoarthritis. Primary repair and reconstruction are representative surgical strategies. ACL reconstruction is widely performed but not all patients experience satisfactory recovery following ACL reconstruction. Therefore, many orthopedic surgeons refocused their attention to ACL primary repair because of its less invasive nature and potential for self-healing. Several inherent advantages exist in ACL primary repair, including fewer graft-related complications and maintenance of the original anatomical structures and proprioceptors. There is an unmet clinically relevant animal model to investigate the efficacy and mechanism of these two surgical strategies. This study aims to establish a novel murine model of ACL healing in primary repair and compare with the reconstruction of proximal ACL tears. The objectives of this study are: (1) to describe a murine animal model of ACL repair after femoral-side ACL rupture and (2) to document the effect of ACL repair compared to the ACL reconstruction following femoral-side ACL rupture. The main perspective is to develop a reproducible and well-characterized femoral-side ACL rupture and repair small animal model that would be available for further studies on healing mechanism, bio-material and advanced treatment intervention for ACL repair.

**SUBJECTS AND METHODS:** This study was approved by Shanghai People's Sixth Hospital IACUC. 40 cadaveric knees from skeletally mature (12-weeks old) male Sprague-Dawley rats were used to practice model establishment. Femoral-side ACL tears was induced in right legs of 144 rats. The animals were divided into five groups by different timepoints (24 rats/group): I: 3 days; II: 1 week; III: 2 weeks; IV: 4 weeks; V: 8 weeks. Another twelve rats were used as controls. (Fig 1) Femoral-side ACL rupture (Fig 2): A standard medial parapatellar arthrotomy was created to obtain the access to the joint. The patella was subluxated laterally and the fat pad over the intercondylar area was subsequently dissected to expose the ACL. 21-gauge needle was stabbed for 5 times and then Axial force was applied manually using the scalper handle along the axis of the femur with the tibia fixed until a distinct "popping" was felt, indicating an ACL rupture. The force was stopped immediately once the ACL ruptured to avoid further damage to the other ligaments. A manual Lachman test, followed by direct joint inspection, was done to verify the femur-side ACL rupture. Primary repair surgery (Fig 2): 5-0 polyester braided suture was used to braid the end of ACL rupture remaining. The femur bone tunnel was drilled at the location of the native ACL footprints in a retrograde fashion using a 23-gauge needle and the tendon-fixed suture passed through the tunnel with the tendon attached the bone tunnel inter orifice. A microvascular clip with three suture knots provided suspensory fixation on the femur side. Specimens were prepared for the following outcomes: (1) Clinical outcomes; (2) Histological analysis; (3) Micro CT evaluation; (4) Biomechanical testing. Statistical analysis: t-tests and One-way ANOVA with Tukey's test were used. The significance limit was set at  $P < 0.05$ .

**RESULTS:** Clinical outcomes: Each animal was observed for its health and activity status daily including the presence of lameness until sacrifice at given timepoints. The reconstruction group at 3 days, 1w and 2w still lameness while the repair group at 3 days and 1w showed lameness and gradually recover from 2 weeks timepoints. Histological evaluation (Fig 3): The H&E staining showed inflammation synovium at early timepoints (3d, 1w, 2w) in both repair and reconstruction group, while the reconstruction group had severer synovitis than the repair group. The inflammation decreased gradually after 4w. (3A) The interface healing process started from 1w in repair group and 2w in reconstruction group both with scarring tissue. At 8w, fibrous tissue was observed in both groups. (3B) The articular cartilage surface injury was observed in both groups from Safranin-O-staining but the reconstruction group had more degeneration. (3C) Picosirius Red staining showed

gradually organized ligament collagen alignment in both groups. (3D) Micro-CT evaluation (Fig 4): The bone volume fraction (BV/TV) increased with the healing process. There is significant difference at 2, 4 and 8 weeks between the two groups. However there has no difference in bone mineral density at all timepoints in both groups. The significantly increased in trabecular number of the repair group compare to the reconstruction group at 2, 4 and 8 weeks timepoints. No difference was demonstrated in connectivity density compare with the two groups. The trabecular space showed increased significance in reconstruction group compare with the repair group at 2, 4, and 8 weeks. Biomechanical testing: The failure force of 8w in both groups showed significant increased compared to the 4w group although no significance was found between the repair and reconstruction group. The stiffness demonstrated the similar trends. (Fig 5)

**DISCUSSION AND CONCLUSION:** Our data showed the different timepoints of healing process following ACL primary repair and reconstruction surgery. These findings would validate this model for further study of ACL primary repair. Considering the unsatisfied long-term efficacy of ACL reconstruction, it may fail because of being unable to restore the normal anatomical structure and physiological function of the torn ACL. Primary repair is based on full consideration of the location, type and quality of ligament tissue of ACL injury. The primary repair had a significantly shorter operative time and lower cost than reconstruction, which could be considered as the benefit of primary repair in treating proximal ACL tears for some specific patients. This novel ACL primary repair murine model would permit further research on ACL repair mechanism and graft healing, maturation, function and more interventions, which will provide guidance of target patients and rehabilitation program following the surgery. A limitation of our study is lack of longer time points to further elucidate the long-term healing process and efficacy of ACL primary repair and reconstruction. In conclusion, a preclinical model of primary repair for femoral-side ACL tear was established for further outcome and mechanism study in ACL regeneration.

## Biomimetic soft-to-hard interface with gradient nanostructure for bone-tendon regeneration

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**Introduction:** A graded fibrocartilage regeneration after tendon-bone injury remains a challenge. As a gradient mineralization tissue, the tendon-bone tissue assembly begins at the ultra-small nanoscale. At present, the biomimetic soft-to-hard interface can't be precisely engineered by using the materials of tens or even hundreds of nanometers, which is hard to restore functionality upon tissue repair.

**Methods:** Here, using type I collagen molecular and amorphous calcium phosphate ( $< 2$  nm), we synthesize a gradient biomineralization-inspired hydrogel.

**Results:** The hydrogel is composed of three regions with distinct functions: (i) a region without mineral to guide integration of the hydrogel with the tendon side, (ii) a region with mineral (34 wt%) to facilitate function transfer between tendon and bone, (iii) a region with mineral (61 wt%) to promote the integration of the hydrogel with the underlying bone. At the nano-scale, fibrils without mineral display the typical 67 nm D-period, while mineralized fibrils have mineral accumulation in both intra- and extrafibrillar compartments, akin to native bone. In vitro experiment, the seeded stem cells exhibit directed differentiation into tenocytes, mineralized fibrochondrocytes, and osteoblasts along the mineralization gradient. Furthermore, four weeks after surgery, the hydrogel could induce considerable fibrocartilage arrangement and ingrowth at the tendon-bone tissue in vivo.

**Conclusion:** This novel hydrogel holds great promise to promote the formation of a functional tendon-bone attachment by offering a structurally and compositionally appropriate microenvironment for healing.

## Type H vessels mediated by PDGF-BB enhances the Tendon-bone healing through modulation of the coupling between angiogenesis and osteogenesis

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Background: Type H vessels have been recently found to closely modulate bone formation via regulation of the osteo-angiogenic crosstalk. As how to promote the bony ingrowth towards the tendon graft-bone interface (TBI) is the one of the critical factors influencing healing quality after anterior cruciate reconstruction in patients, the strategies favoring type H vessel formation may be promising therapeutic approaches aiming at improving the tendon graft osteointegration into bone tunnels.

Methods: A total of 87 ten-week-old male mice underwent ACL reconstruction were randomly assigned into three groups: treatment with hydrogel microparticles (HMP, set as the control group), treatment with slit3 loaded HMP (slit3@HMP), and treatment with slit3 neutralizing antibody loaded HMP (slit3-AB@HMP). The knees of mice (n=5/group/time point) at 2, 4, and 6 weeks post-surgery were then harvested for general histological and immunofluorescent analyses and micro-computed tomography (micro-CT) scanning and assessment. The remaining 6 mice in each group were euthanized at 4 and 6 weeks for measurement of dynamic knee joint laxity and load to failure in each femur-graft-tibia complex. The mice (n=6/group) in the above three groups assigned for biomechanical testing at 6 weeks post-operation and the mice in the sham group (n=6) were used for gait analysis at 1, 2, 4, and 6 weeks after surgery.

Results : Increased bony ingrowth while reduced fibrous scar tissue was formed at the TBI in the slit3@HMP group when compared to the control group. Meanwhile, the slit3-AB@HMP inhibited the osseous ingrowth and increased fibrous scar tissue formation at the TBI relative to the HMP alone. Importantly, compared to the control group, the slit3@HMP treatment favored type H vessel (CD31<sup>hi</sup>Emcn<sup>hi</sup>) formation at the TBI while the slit3-AB@HMP treatment showed the opposite results. According to micro-CT assessment, compared to the control group, the slit3@HMP treatment significantly increased the peri-tunnel bone mass while the slit3-AB@HMP treatment significantly reduced the peri-tunnel bone mass. In gait analysis, the mice in the slit3@HMP group showed best performance relative to the other two groups in terms of stance time, stride length, paw print area, and stance pressure. Dynamic knee joint laxity measurement and tensile testing showed that the slit3@HMP group exhibited significantly reduced displacement of the tendon graft and improved load to failure and stiffness relative to the other two groups.

Discussion and conclusion : This is the first study to investigate the effects of slit3 treatment on the tendon-bone healing through modulation of type H vessel formation at the TBI in mice models with ACL reconstruction. Increased CD31<sup>hi</sup>Emcn<sup>hi</sup> endothelium was observed at the TBI after slit3 treatment while the opposite result was detected when the slit3-AB was applied in the murine models. Concomitantly, the slit3 treatment favored the bony ingrowth towards the TBI, contributing to improved gait performance and load to failure while less laxity displacement. The results indicated that the local injection of slit3 may be a promising approach to accelerate and enhance the graft osteointegration into bone tunnels in patients with ACL reconstruction.

## Magnesium facilitates the consolidation of distraction osteogenesis: A innovative strategy for enhancing the repair of large segmental bone defect

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**Background:** The management of large bone defects is a global challenge in orthopedics and trauma. Distraction osteogenesis (DO) is effective for the treatment of large bone defects. However, the long treatment duration of DO limits its clinical application. Recently, magnesium (Mg) metal degradation has been reported to promote new bone formation in the early consolidation phase of DO, but its effectiveness in promoting bone remodeling at the late stage of consolidation and thus shortening the treatment duration of DO remains unknown. As intraosseous vessels, type H vessels play an important regulatory role in bone remodeling. Our preliminary results revealed that Mg promoted type H vessel formation and promoted bone remodeling during the consolidation phase.

**Methods:** Herein, we developed an innovative intramedullary biodegradable magnesium (Mg) nail to accelerate bone regeneration in large-size bone defect repair during DO. In this study, we clarified that Mg shortens the DO consolidation period via mechanical assessment and histology assessment. We identified the key factor involved in Mg-promoted type H vessel formation through molecular biology methods. Furthermore, single-cell sequencing was applied to reveal the key pathway and factors that regulate Mg-promoted type H vessel formation.

**Results:** Our results revealed that magnesium promoted type H vessel formation, upregulated integrin expression, and promoted bone remodeling during the consolidation phase, and single-cell sequencing results suggested that the integrin pathway is a potential target for Mg-promoted type H vessel formation.

**Discussion and Conclusion:** This study revealed the mechanism of Mg-promoted type H vessel formation and accelerate the clinical translation of Mg for the treatment of large bone defects.

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## Non-woven hydrogel scaffold loaded with quercetin for airway epithelial injury repair

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Title: Non-woven hydrogel scaffold loaded with quercetin for airway epithelial injury repair

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Category: Tissue Engineering and Regeneration

Background: The airway epithelium, through its surface mucus and apical complex junctions, forms an important physiological barrier to the internal and external airway environment and maintains the homeostasis of the lung environment by performing scavenging functions and producing bioactive components. However, the airway epithelium itself is highly susceptible to damage by the external environment, so a quercetin non-woven hydrogel scaffold with airway epithelial repair function was constructed for airway epithelial repair.

Methods: In this work, quercetin-loaded hydrogel-based non-woven materials were prepared using wet web formation and freeze-drying, and reinforced with filamentous proteins on the surface. The non-woven hydrogel scaffold was then applied to the injured airway surface to modulate the local inflammatory microenvironment and induce migration and proliferation of airway epithelial cells.

Results: As the concentration of spinning solution increases during preparation, the fibre surface becomes smoother and quercetin is encapsulated within the fibre (Figure A & B). The material is biocompatible (Figure C,D&E) and protects epithelial cells under oxidative stress and promotes their proliferation and migration (Figure F-K). We subsequently found that quercetin hydrogel fibre scaffolds alter the oxidative stress environment through ROS scavenging (Figure L-O) and activate epithelial stemness and promote epithelial migration and regeneration in a rabbit epithelial injury model (Figure P&Q).

Discussion and Conclusion: We demonstrate that non-woven hydrogel scaffold loaded with quercetin protect epithelial cells by scavenging intracellular ROS in an oxidative stress environment and promote epithelial migration and regeneration in vivo, thereby repairing damaged airway epithelium.



## Activation of CGRP Receptor-mediated Signaling Promotes Tendon-bone Healing through modulation of osteogenesis and re-innervation

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Category: SYIS (Student and Young Investigator Section), designed and application of biomaterials.

Introduction: Angiogenesis and innervation are found to display potential role in promotion of osseous ingrowth towards the tendon-bone interface due to the driving forces of modulating bone regeneration. As the neurotransmitter calcitonin-gene related peptide (CGRP) displayed positive osteopromotive role in bone regeneration[1], the adenovirus-delivered shRNA or cDNA (targeting Calcrl, CGRP receptor) loaded into the hydrogel microparticles (HMP) was applied to modulate CGRP signaling pathway in the tendon-bone healing. Herein, we hypothesized that the CGRP signaling pathway may regulate Type-H blood and nerve fiber regeneration at the tendon-bone interface (TBI), thereby enhancing the graft integration.

Materials and Methods: For the in vitro study, the mouse derived BMSCs were cultured with osteogenic-induced medium containing CGRP with and without H-89 (PKA inhibitor) prior to RT-qPCR and western blot analysis. For the in vivo study, anterior cruciate ligament (ACL) reconstruction was performed in the C57BL/6J male mice, which were divided into three groups including PBS, HMP-adv-shCalcrl and HMP-adv-Calcrl treatment. Gait performance, biomechanical testing, radiographic measurement, and histological analysis were performed to assess the TBI healing in mice euthanized at 2-, 4-, and 6-weeks post-surgery (n=6/group/time point). One-way ANOVA with Tukey's post-hoc correction was used for multiple comparisons of data in over two groups (p<0.05 was set as significant difference).

Results and Discussion: In vitro studies revealed that CGRP enhanced osteogenic ability of BMSCs through PKA/CREB/JUNB pathway, contributing to increased levels of sonic hedgehog (SHH) beneficial for nerve regeneration. Consistently, overexpression of CALCRL (adv-Calcrl) dramatically favored osteointegration of the tendon graft as well as an increase CGRP+NF200+ nerve fibers, Shh and number of type H (CD31hiEMCNhi) vessels while knock-down of CALCRL (adv-shCalcrl) remarkably inhibited bony ingrowth and nerve regeneration and decreased the formation of type H vessels at the tendon graft enthesi in bone tunnels. In addition, gait analysis, radiographic measurement and biomechanical testing further demonstrated significantly improved tendon-bone healing quality for the adv-Calcrl group compared to the control group, while the adv-shCalcrl group showed the opposite results. These results showed improved gait characteristics and biomechanical performance.

Conclusion: We prepared a novel microhydrogel to encapsulate adenovirus for gene therapy, with an extended while more stable in situ release, aiming to modulation of CGRP receptor expression levels in bone tunnels. Encouragingly, the adv-Calcrl treatment significantly favored the formation of CGRP+ sensory nerve fibers and CD31hiEMCNhi endothelium at the TBI, demonstrating that the activation of CGRP receptor-mediated signaling is a potential therapeutic strategy to improve healing quality in patients with ACL reconstruction.

### References

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Acknowledgement (optional)

## Industrialization of Progenitor Cellular Therapies in Modern Musculoskeletal Plastic Surgery

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**Background:** Scientifically sound cell-based regenerative medicine has never been stronger with “living cell therapy”. Respective progress made in bioengineering and in biotechnology have enabled the clinical implementation of modern cell-based therapeutic treatments within updated medical and regulatory frameworks. Notably, the Swiss progenitor cell transplantation program has enabled the gathering of over two decades of clinical experience in Lausanne for the therapeutic management of diverse cutaneous and musculoskeletal affections, using homologous allogeneic cell-based approaches.

**Methods:** Research was oriented for optimization of cell selection techniques, specific cell-based therapy formulation, and procedures for direct use in the clinic. Prenatal tissue was obtained under a Federal Transplantation Program umbrella (skin, tendon, cartilage, bone, lung and disc tissues). FE002 progenitor cells were cultured within 2 weeks for multi-tiered cell banking purposes. Initial clinical trials using living progenitor skin cells as novel therapeutic cell sources began in 2000 with “progenitor biological bandages”, used for pediatric burn patients and later for chronic wounds in adults. Further development of cell-based formulations (e.g., lysates, sterilized lyophilizates) for skin, tendon, bone, lung, disc, and cartilage affections is underway (i.e., focus on bioactivity preservation and regulatory compliance).

**Results:** Following worldwide regulatory updates in 2007, a new Swiss program of Transplantation for Musculoskeletal Tissues was set up, along with formal registration and an associated Biobank Program. Translational focus on musculoskeletal progenitor cell banks has enabled to successfully produce human bone, cartilage, disc, muscle, tendon, and skin cell sources under R&D and GMP conditions. Progenitor biological bandages, formulated following the new Directives and with registered clinical cell banks, have been used routinely in the clinic since 2004, particularly for severe burns in both pediatric and adult patients. It was further shown (industrial preclinical testing and GLP animal models) that specific progenitor cell sources (derived from cartilage, bone, disc, and tendon) can be optimized for therapies with enhanced biological properties through various scaffold associations, for potentially enhanced therapeutic management of diverse musculoskeletal affections.

**Discussion and Conclusion:** Establishing unique and extensive musculoskeletal progenitor cell banks has supported the core belief that cell therapies will overtake classical pharmaco-therapeutic management protocols for numerous traumatic and degenerative pathologies. Clear advantages of allogeneic progenitor cells reside in the enhanced and highly specific stimulation of damaged tissues toward restored health and function.

## Targeting and selecting high cytokine-secreting cells for regenerative medicine

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### Background:

Probing how cells secrete cytokines as they respond to the surrounding signals is a major challenge. Given the important roles of cytokines across the biological spectrum, including the control of cell replication and apoptosis, cancer, atherosclerosis, and tissue regeneration and in the modulation of immune reactions, it is critical to advance the understanding of the heterogeneity of cellular cytokine release at the level of single cells. This inspired us to create a simple and sensitive single-cell cytokine analysis platform that enables a nuanced characterization and selection of individual high cytokine-secreting cells as well as quantitative analysis of cytokines secreted from each cell

### Methods:

we present a universal approach to highly sensitive detection of trace cytokine secretions from individual, single live cells, by utilizing the cell surface to capture the secreted molecules where they can be detected by fluorescent magnetic nanoparticle labelling. The capture surface was created by fabricating a sandwich biosensor on the cell membrane, that do not affect cell secretion and enable proliferation.

### Results:

We developed a universal method to detect cytokine secretion from individual cells by applying a capture technology on the cell membrane. The assay uses fluorescent magnetic nanoparticles as assay reporters that enable detection on a single-cell level in microscopy and flow cytometry and fluorimetry in cell ensembles. This system is flexible and can be modified to detect different cytokines from a broad range of cytokine-secreting cells. We have been able to select and sort highly cytokine-secreting cells and identify cytokine-secreting expression profiles of different cell populations *in vitro* and *ex vivo*. We show that this system can be used for ultrasensitive monitoring of cytokines in the complex biological environment of atherosclerosis that contains multiple cell types. The ability to identify and select cell populations based on their cytokine expression characteristics is valuable in a host of applications that require the monitoring of disease progression.

Scheme shows the cell membrane sensor is able to identify and select of high cytokine-secreting cells.

### Discussion and Conclusion

With these new capabilities of cell surface biosensor, we were able (1) to assess the ability of individual cells to secrete cytokines, (2) to distinguish highly secreting cells from poorly secreting ones, and (3) aided by fluorescence *in situ* hybridization labeling of the relevant messenger RNA, to provide insights into the cytokine secretion dynamics, in particular on the existence of early and late responders to cytokine stimulation. Furthermore, brightly fluorescent magnetic bead labeling made it possible to detect the *ex vivo* secretion of IL-6 from multi-cellular atherosclerotic plaque-containing mouse aortae. This cell membrane assay was responsive to an inflammatory stimulus and to an increase in the stage of atherosclerotic disease development. The capability to select specific cells with a range of cytokine secretion levels and the ability to purify cell populations through identification of cellular expression levels on a single-cell basis may have significant implications for future cell therapy applications and for tracking disease progression in preclinical models.

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## Oxygenating materials for tissue regeneration and stem cell fate commitment

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Title: Oxygenating materials for tissue regeneration and stem cell fate commitment

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Category: Design and Application of Biomaterials

Background: Oxygenating biomaterials have been developed to alleviate anoxic stress, stimulate vascularization, and improve the engraftment of cellularized implants. However, the effects of self-oxygenation materials on different aspects of regenerative medicine such as healing of the myocardium after myocardial infarction (MI) or stem cell fate in cellularized hydrogels have not yet been studied.

Methods: In this study, oxygenating biomaterials were mixed with different hydrogels for two different applications: 1.) Tissue regeneration in MI and 2.) Stem cell fate commitment for regenerative medicine such as bone regeneration (Schematic 1). Calcium peroxide as the source of oxygen was encapsulated in polycaprolactone to produce oxygenating microparticles with prolonged oxygen release profiles. These oxygen-generating microparticles were incorporated into bioadhesive silk-alginate or gelatin methacryloyl (GelMA) based hydrogels with and without mesenchymal stem cells (MSCs), for MI and osteochondral differentiation, respectively. For MI, these hydrogels were conjugated with stromal cell derived factor (SDF) to orchestrate chemotaxis and angiogenesis and generate oxygen via oxygenating microparticles (OMPs) to alleviate the cytotoxic anoxic environment. Silk fibroin (SF) was explored to endow elasticity and resilience to the injectable hydrogel. Additionally, tyramine (TA) was conjugated to alginate (TA-Alg) and SF, producing a mechanically robust and tissue adhesive hybrid hydrogel (TSF) that encourages tissue adhesion and enhances the injectability of the hydrogels.

For stem cell fate commitment for bone tissue engineering applications, oxygen-generating microparticles were incorporated into GelMA hydrogels in the presence/absence of osteoinductive silicate nanoparticles (SNPs). A comparative study revealed the osteogenic fate of hMSCs in the designed hydrogels under normoxic (the gold standard for in vitro cultures) and anoxic (common in large bone defects; <0.1% oxygen) conditions.

Results and Discussion: The hydrogel was shown to continuously release SDF and oxygen for MI applications. The subsequent combinational and synergistic effect results in a significant improvement in vascularization, increasing the cardiomyocyte survival by 30% cardiomyocyte survival and reducing the fibrotic scar formation in an MI animal rodent model. While TSF with OMPs hydrogels perform better than pristine hydrogels, combination with SDF significantly improves the left ventricular systolic and diastolic function by approximately 10% and 20%, respectively, and leads to a significantly higher maintenance of ~ 25% higher ejection fraction at day 7 compared with animals treated with pristine TSF hydrogels.

To study the role of oxygenating biomaterials in driving fate commitment in MSCs, in line with the literature, under standard laboratory conditions (normoxia), stem cells exposed to osteoinductive SNPs most effectively were differentiated into bone-forming osteoblasts. However, under in vivo-like conditions (anoxia), OMPs were demonstrated to be highly osteoinductive; in fact, significantly more osteogenesis was observed in OMPs than in the commonly used SNPs condition. This is highly surprising and has significant clinical relevance as it represents the first material that offers both oxygenation and osteoinduction, both of which are essential processes to achieve successful bone defect healing. Bulk RNA-sequencing confirmed that OMPs possessed a stronger expression of osteogenic gene transcript fingerprint than SNPs or SNPs in combination with OMPs. The addition of OMP also increased the number of vessels and potentially increased vessel diameter and thus vessel

maturation. The study presented in this submission reflects on the role of oxygenating biomaterials in osteogenesis for regenerative medicine applications required under oxygen-deprived circumstances such as bone tissue scaffolds. Innovatively, we here demonstrate that endowing hydrogels with oxygen-generating microparticles can induce, improve, and steer the formation of functional engineered living tissues, which holds potential for numerous biomedical applications, including tissue regeneration and organ replacement therapy.

**Conclusion:** A combinatorial effect of OMPs and SDF prevented hypoxia induced cell death in vivo and created vessel networks within the implants as demonstrated by immunofluorescence and optical clearing of the heart tissue around the treated MI injury area. Our approach demonstrates that the sustained release of exogenous oxygen and SDF with the substantial diffusion capacity and mechanical properties of TSF injectable hydrogels was able to favor a significant improvement in tissue vascularization after myocardial injury, thus promoting cardiomyocyte survival and limited fibrotic scar formation. A histologic analysis revealed that these therapeutic hydrogels led to an increase in angiogenesis and vasculogenesis at the infarct site, in addition to a reduction of the scar tissue and decreased number of dead cells at the injury.

In the second part of the study, we report that endowing biomaterials with self-oxygenating properties can promote the osteogenic fate of encapsulated hMSCs. We investigated hydrogel scaffolds reinforced with OMPs for their oxygen releasing capabilities and their influence on driving osteogenesis in vitro and in vivo under different environmental conditions. A comparison of effects of OMPs with and without an osteoinductive mineral, SNP, was made for mechanical resilience of the scaffolds, cell survival, and expression of several bone development markers under normoxic and anoxic conditions. In vitro, OMPs alone showed higher levels of cell survival and proliferation, but SNP addition resulted in higher ALP activity. Bulk RNA studies revealed that OMPs induced a more osteogenic gene transcript fingerprint than SNPs or SNPs and OMPs combined. In vivo, self-oxygenation of biomaterials demonstrated a greater host cell invasion that correlated with enhanced vasculogenesis.

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