## Patient-derived organoids to model frozen shoulder disease

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**INTRODUCTION:** Diseases affecting the musculoskeletal system present a considerable global health burden, causing pain and disability and increasing the likelihood of developing metabolic comorbidities. Translational research towards addressing this issue uses animal models to great effect in uncovering the mechanisms of MSK morbidities such as osteoarthritis and fracture healing. Frozen shoulder is unlike other MSK diseases in that it is frequently self-limiting, and thus represents a promising research area towards encouraging fibrosis resolution in other joints. However, current animal models do not recapitulate frozen shoulder pathology due to anatomical differences, necessitating an *in vitro* approach. Current work on 3D cell culture, namely organoids, has proven translationally applicable in several visceral soft tissues, including gut and lung. Given this, and the challenges inherent to 2D *in vitro* experiments, we hypothesize that developing a 3D patient-derived organoid (PDO) model of frozen shoulder is a feasible approach to address mechanistic questions about the disease's unique resolution arc.

**METHODS:** Creation of patient-derived organoid models of arthrofibrosis. The three cell types comprising our PDO (fibroblasts, macrophages, and HUVECs) were cultured and combined as follows: Fibroblasts were derived from explant culture using either diseased frozen shoulder or comparator capsule tissues, and used at P2-3. Monocytes were isolated from pooled blood donors and differentiated into macrophages (M0) in 2D culture. HUVECs were routinely cultured and used at P4-6. All cell types were resuspended together in Matrigel and seeded in droplets onto low adhesion plates. After 2-3 days PDOs detach from the plate and cultured in suspension with frequent media changes.

*Immunofluorescence*: PDOs were processed for paraffin and cut in 5µm sections. Slides were probed with antibodies against Ki67, CD146, and nuclear stain POPO-1, followed by confocal imaging. Corresponding sections were stained with H&E to visualize organoid morphology.

Whole organoid imaging: PDOs were fixed and permeabilized prior to incubation with DKK3 to mark fibroblasts, CD31 to stain HUVECs, and POPO-1 as nuclear stain. Confocal z-stack imaging was performed to determine potential HUVEC tube formation.

**RESULTS:** Frozen shoulder organoids exhibit a thickened, proliferative lining layer in contrast to comparator shoulder organoids. Confocal imaging of stained PDO sections revealed the PDOs form a characteristic lining and sub-lining region resembling synovial tissue, with the frozen shoulder organoid displaying a thickened lining layer similar to that seen in diseased shoulder synovium (**Fig. 1A**). Increased CD146 staining was observed in this thickened lining region, along with areas of Ki67, suggesting elevated fibroblast activation and proliferation in the frozen shoulder organoids (**Fig. 1B**).

Frozen shoulder organoids demonstrate HUVEC tube formation by D10 culture. Whole organoid z-stack imaging showed even distribution of HUVECs throughout the organoid, with networks reminiscent of tube formation visible (Fig. 2A). 3D projection of HUVECs in comparator vs frozen shoulder PDO reveals a better defined HUVEC network present in frozen shoulder PDOs, mirroring the increased vascularization seen in the disease (Fig. 2B).

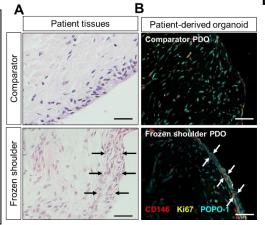
**DISCUSSION**: Patients with frozen shoulder syndrome demonstrate a thickened lining capsule layer as part of the disease pathology, along with increased vascularity and inflammatory processes. We have shown here that organoids cultured with comparator- vs frozen shoulder-derived fibroblasts recapitulate several diagnostic markers seen clinically. Fibroblasts, macrophages, and endothelial cells self-organize into PDO that emulate the distinctive lining and sublining regions seen in patient shoulder capsule tissues, with the frozen shoulder PDO exhibiting increased lining thickness and vascularity compared to healthy. Interestingly, we found that while HUVECs formed tubes quickly when cultured alone on the surface of Matrigel in 2D, they did not when seeded within the Matrigel; this suggests that HUVEC tube formation within the shoulder PDOs may be due to crosstalk between the co-cultured cell types. Therefore, this shoulder PDO model may present a novel way to interrogate cell-cell crosstalk initiating and maintaining disease processes, and a viable model to test potential therapeutic interventions.

**SIGNIFICANCE:** Here we have developed a 3D patient-derived organoid model to recapitulate frozen shoulder syndrome, representing a viable approach to better understand why this disease resolves and potentially uncover targets to treat other types of arthrofibrosis.

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**REFERENCES**: PA Johnson, JE Ackerman, M Kurowska-Stolarska, M Coles, CD Buckley, SG Dakin. Three-dimensional, in-vitro approaches for modelling soft-tissue joint diseases. The Lancet Rheumatology 2023 Sept. e553-e563. doi: 10.1016/S2665-9913(23)00190-X.

Frozen Figure 1. shoulder **PDOs** recapitulate cellular arrangement seen in patient tissues. A: H&E stained sections of patient synovial tissues taken from a comparator or frozen shoulder. B: D10 triorganoids stained with CD146 (red), Ki67 (yellow), and POPO-1 (cyan). Arrows thickened indicate lining layer.



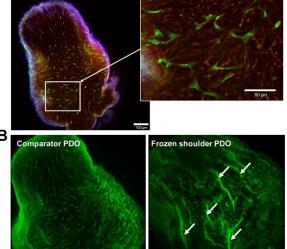


Figure 2. Endothelial cell tube formation observed in shoulder PDOs. A: whole organoid imaging demonstrating distribution of HUVECs (CD31, green), and fibroblasts (DKK3, red), with nuclear stain POPO-1 (blue). B: 3D projections of PDOs reveal increased HUVEC tube formation in frozen shoulder PDO (arrows).