Differentiation and Functional Characterization of Human iPSC-Derived Fibroblast-Like Synoviocytes

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INTRODUCTION: Fibroblast like synoviocytes (FLS) play a central role in joint pathology by coordinating leukocyte recruitment and driving pannus invasion that erodes cartilage in inflamed synovial joints. They are a major source of inflammatory mediators and matrix degrading enzymes in rheumatoid arthritis (RA) and osteoarthritis (OA) [1]. Consequently, establishing robust protocols for differentiating human iPSC-derived-FLS (iFLS) is essential for building isogenic, patient-specific "joint-on-a-chip" to create human-centric *in vitro* models of RA and OA and to evaluate potential disease-modifying therapeutics. The use of iPSC enables genotype matched stromal cells across compartments, isogenic editing to test causal variants, and ensures ample supply of cells for reproducible studies. Building on a published murine protocol [2], the objectives of this study were to establish a human iFLS differentiation protocol, characterize iFLS identity through immunostaining in comparison with primary FLS, and benchmark iFLS functionality against primary FLS using scratch closure assays with and without inflammatory TNF-α stimulation. We hypothesized that iFLS would acquire synovial features (PRG4+, CDH11+), express canonical FLS markers (CD90, CD55, PDPN, αSMA), and exhibit functional behaviors resembling primary FLS, including proliferative and migratory responses after inflammatory TNF-α-stimulation.

METHODS: Human iPSCs, reprogrammed from tendon fibroblasts obtained from tenolysis tissue under approved IRB protocols, were differentiated to fibroblast-like synoviocytes (iFLS) by adapting a published murine protocol [2]. On Day 0, single-cell iPSCs were plated at 15 × 10³ cells/cm² on Matrigel-coated plates in mTeSR Plus with Y-27632 (10 μM). From Days 1–4, cells were induced toward mesodermal lineage with daily medium changes. On Day 5, differentiation was initiated using FLS differentiation medium (MesenCult ACF Basal supplemented with 2mM L-glutamine, 10 ng/mL TGF-β1, and 10 ng/mL bFGF), which was refreshed every 2–3 days until Day 21. Cells were then harvested, cryopreserved (10% DMSO), and recovered in FLS medium for 7 days prior to assays. Primary FLS for were maintained as benchmarking controls in DMEM + 10% FBS + 1% penicillin/streptomycin. Primary and induced FLS phenotype was assessed by immunostaining for PRG4, CDH11, CD90, and CD55. Immunostaining for PDPN and α-SMA and scratch wound assays were performed on confluent iFLS and primary FLS (cultured in DMEM + 1% FBS + 1% penicillin/streptomycin) with or without TNF-α stimulation (10 ng/mL). Wounds were imaged at 0, 4, 8, and 24 h, and percent wound closure was quantified in Fiji (ImageJ). Statistics: Data from three independent experiments (n = 3) were analyzed by two-way ANOVA, with Bonferroni-corrected multiple comparisons between iFLS and primary FLS responses.

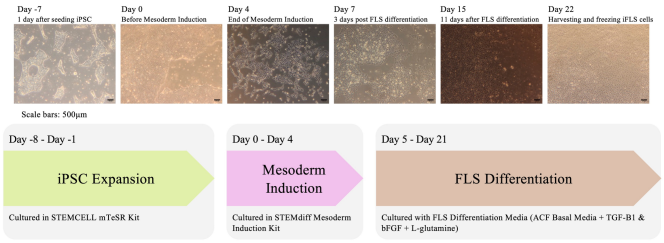

Fig.1 Timeline of iPSC differentiation into iFLS demonstrating stepwise morphological changes.

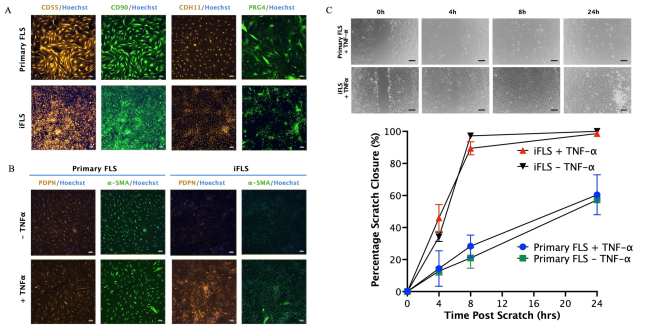

Fig.2 Characterization of primary FLS and iPSC-derived fibroblast-like synoviocytes (iFLS). **(A)** Immunofluorescence staining of CD55, CD90, CDH11, and PRG4 in primary FLS and iFLS under basal conditions.**(B)** Immunofluorescence staining of PDPN and α-SMA in primary FLS and iFLS with or without TNF-α stimulation (10 ng/mL)**.** Scale bars =100 microns. **(C)** Quantification of scratch wound closure in primary FLS and iFLS with or without TNF-α stimulation over 0–24 h (n = 3). Data were analyzed by two-way ANOVA; $p < 0.001$ for comparisons between iFLS and primary FLS at all time points. Scale bars =100 microns.

RESULTS: Both primary FLS and iFLS expressed characteristic synovial markers, including CD55, CD90, CDH11, and PRG4, however, compared with primary FLS, iFLS appeared smaller and more compact, with higher cell density across all markers examined (Fig. 2A). Primary FLS exhibited larger, elongated morphologies with more pronounced spreading, whereas iFLS displayed a smaller, less spread morphology. PDPN and α-SMA expression was robust in both primary and iFLS upon TNF-α stimulation (10 ng/mL), consistent with an activated phenotype. However, while primary FLS expressed basal levels of PDPN and α-SMA, iFLS did not express these activation markers in the absence of TNF-α stimulation (Fig. 2B). Scratch closure assays revealed significant differences in proliferative and migratory rates between iFLS and primary FLS. iFLS exhibited accelerated wound closure, achieving >80% closure within 8 hours under both basal and TNF-α stimulated conditions, whereas primary FLS showed slower would closure kinetics (Fig. 2C) regardless of TNF-α treatment. Subtle acceleration in wound closure was observed at 4h under TNF-α stimulation in both cell types, but by 8 and 24h, precent closure converged. These findings demonstrate that iFLS recapitulate key phenotypic markers of primary FLS but display distinct morphologic characteristics and significantly enhanced migratory activity, particularly in response to TNF-α.

DISCUSSION: Fibroblast-like synoviocytes are central drivers of joint inflammation and tissue destruction in RA and OA. Our results show that iPSC-derived fibroblast-like synoviocytes (iFLS) reproduce many primary FLS phenotypes, expressing key markers and responding to TNF-α with upregulation of PDPN and α-SMA. iFLS also maintained robust PRG4 and CD90 expression and demonstrated enhanced migratory capacity, resembling the aggressive, invasive subset of RA synovial fibroblasts that drive pannus formation and cartilage destruction under inflammatory conditions [3]. Morphologically, primary FLS were noticeably larger than iFLS, underscoring that iFLS are not identical to their primary counterparts. It is possible that the expansion of primary fibroblasts affected their basal expression of activation markers. It is also conceivable that iFLS, which were obtained from pathologic tenolysis fibroblasts, may have retain epigenetic memory of their fibrotic progenitors. Future studies will require additional assays including single cell RNA sequencing to benchmark these iFLS against published data sets of healthy and diseased primary FLS. Nonetheless, generating a renewable population of FLS-like cells that capture core synovial features offers significant value for disease modeling.

CLINICAL RELEVANCE: Patient-derived iPSC fibroblast-like synoviocytes provide a hitherto unavailable human cell source for modeling rheumatoid arthritis and osteoarthritis pathologies. This renewable cell source may be used in patient-specific joint-on-a-chip new approach methodologies (NAMs) to accelerate the development and testing of disease-modifying therapies.

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