Innervation Induces a Distinct Force–Frequency Phenotype in 3D Human Neuromuscular Junction Tissues

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INTRODUCTION: Neuromuscular junction (NMJ) dysfunction underlies many devastating neuromuscular diseases, emphasizing the need for physiologically relevant human NMJ models to study disease mechanisms, evaluate candidate therapeutics, and support potency testing of neuroactive compounds. Conventional 2D coculture systems lack the structural complexity necessary to recapitulate native neuromuscular physiology, whereas 3D engineered tissue models provide a more biomimetic architecture and enable quantitative, force-based functional readouts. Here, we present a human induced pluripotent stem cell (hiPSC)-derived 3D NMJ model that enables longitudinal, noninvasive assessment of contractile force. Interestingly, while embedding motor neurons within 3D skeletal muscle (SkM) improves biochemical and contractile properties in primary cell-derived models,[1-2] comparable benefits in hiPSC systems remain unexplored. Therefore, our objective was to assess whether hiPSC-derived motor neuron innervation drives functional remodeling of the underlying hiPSC-derived skeletal muscle in our NMJ model, which we hypothesized would manifest as changes in contractile response to electrical stimulation.

METHODS: 3D NMJ and skeletal muscle (SkM)-only control tissue models were generated from hiPSC-derived myoblasts and motor neurons on Curi Bio's Mantarray platform following established protocols (**Figure 1A**).[3] Briefly, on day −1, myoblasts and primary human stromal cells were cast into a fibrin-based hydrogel suspended between two flexible posts to form contractile SkM tissues. On day 11, pre-differentiated iPSC-derived neurospheres were incorporated into mature SkM tissues using a collagen/Matrigel overlay to generate NMJ constructs, while SkM-only controls received hydrogel overlay without neurospheres. Tissues were maintained in NMJ maintenance media (Curi Bio) under standard culture conditions (37 °C, 5% CO_2), and contractility was assessed longitudinally from Day 17 to Day 31 using the Mantarray magnetic sensing system. Electrical stimulation was applied via biphasic pulses (5 V amplitude, 100 mA current, 5 ms pulse width) across frequencies ranging from 1–100 Hz to generate twitch and force–vs–frequency (FVF) curves. Force data were collected every 2–3 days from n = 3 tissues per condition across two independent experiments. Data analysis was performed using custom software (Curi Bio) and included quantification of twitch and tetanic force, calculation of the tetanus-to-twitch ratio, and determination of Frequency50 (the stimulation frequency eliciting 50% of maximal tetanic force) by fitting the FVF ratio to a four-parameter logistic function.

RESULTS: While twitch and tetanus forces were comparable between NMJ and SkM-only tissues throughout the culture period (**Figure 1B-C**), NMJ tissues exhibited a distinct FVF plateau above 50 Hz, a phenotype absent from SkM-only controls (**Figure 2A**). By Day 31, NMJs showed a 72% reduction in Frequency50 and a 42% decrease in tetanus-to-twitch ratio relative to controls (**Figure 2B-C**).

DISCUSSION: This study demonstrates a robust, previously unreported divergence in the force-frequency phenotype between hiPSC-derived SkM-only and NMJ tissues. The emergence of an innervation-dependent FVF plateau suggests that neuronal input fundamentally alters excitation–contraction coupling dynamics in human 3D muscle tissues. We hypothesize that this differential FVF response may be due to a shift towards more mature myosin heavy chain isoform and ion channel expression. Our ongoing work will characterize the transcriptomic profiles of these 3D engineered tissues through RNA-seq analysis.

SIGNIFICANCE: By capturing an innervation-dependent contractile phenotype in a scalable, human iPSC-based platform, this work establishes a new functional benchmark for in vitro NMJ modeling. The ability to quantify neuro-muscular coupling and frequency responsiveness positions this system as a powerful tool for high-throughput therapeutic screening, disease modeling, and potency testing of neuroactive compounds.

REFERENCES: 1. Martin et al., Tissue Eng Part A, 2015; 2. Larkin et al., In Vitro Cell Dev Biol Anim, 2006; 3. Fleming et al., CRTOX, 2025;

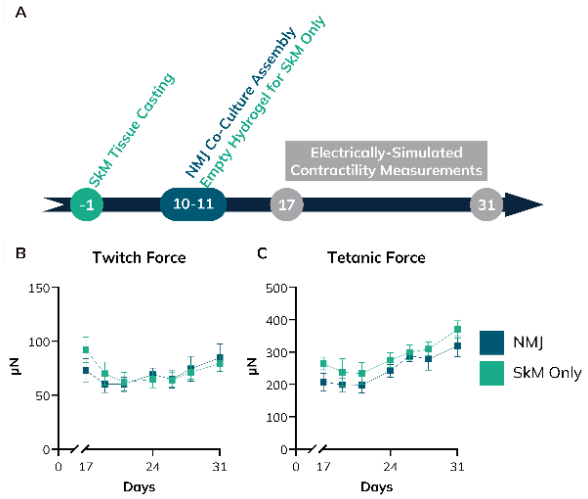

Figure 1. NMJs and SkM only tissues demonstrate similar contractile force magnitudes. (A) NMJs and SkM only controls were generated in two independent experiments (n=3 tissues/group/experiment) and assayed from day 17 to 31. SkM only controls were generated by encasing the 3D SkM tissue in an acellular collagen/Matrigel hydrogel at day 11. (B) Twitch and (C) tetanic force were comparable between NMJs and SkM only tissues across all time points. Data shown as mean ± SEM.

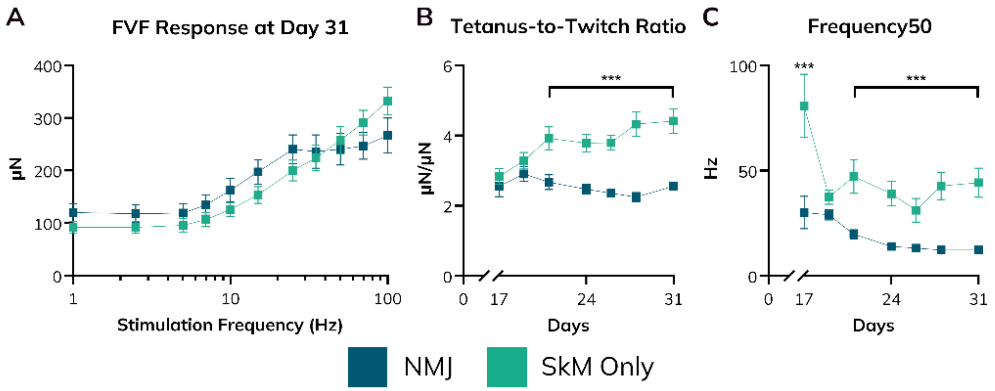

Figure 2. **Innervation drives a specific FVF phenotype in 3D NMJs.** (A) FVF comparison at day 31, demonstrating a plateaued response above 50 Hz in NMJs. (B) Tetanus-to-twitch ratio and (C) Frequency50 were significantly reduced in NMJs from days 21 through 31. Data shown as mean ± SEM. Bars over datapoints indicate statistical significance at that time point (t-test; ***p<.001).