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Tissue engineering the mechanosensory circuit of the stretch reflex arc with human stem cells: Sensory neuron innervation of intrafusal muscle fibers

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Abstract

Muscle spindles are sensory organs embedded in the belly of skeletal muscles that serve as mechanoreceptors detecting static and dynamic information about muscle length and stretch. Through their connection with proprioceptive sensory neurons, sensation of axial body position and muscle movement are transmitted to the central nervous system. Impairment of this sensory circuit causes motor deficits and has been linked to a wide range of diseases. To date, no defined human-based in vitro model of the proprioceptive sensory circuit has been developed. The goal of this study was to develop a human-based in vitro muscle sensory circuit utilizing human stem cells. A serum-free medium was developed to drive the induction of intrafusal fibers from human satellite cells by actuation of a neuregulin signaling pathway. Both bag and chain intrafusal fibers were generated and subsequently validated by phase microscopy and immunocytochemistry. When co-cultured with proprioceptive sensory neurons derived from human neuroprogenitors, mechanosensory nerve terminal structural features with intrafusal fibers were demonstrated. Most importantly, patch-clamp electrophysiological analysis of the intrafusal fibers indicated repetitive firing of human intrafusal fibers, which has not been observed in human extrafusal fibers.

Keywords

Intrafusal fiber; Human; Proprietary sensory circuit; Stem cells; In vitro; Serum-free medium

1. Introduction

Proprioception is the sensation of axial body position and the awareness of limb and body movement through space. Muscle spindle fibers, or muscle mechanoreceptors, are small

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encapsulated sensory organs that lie in parallel with skeletal (extrafusal or contractile) muscle fibers. While extrafusal muscle fibers generate force via muscle contraction to initiate skeletal movement, intrafusal fibers serve as musculoskeletal sensory organs to detect the amount and rate of change of muscle length and monitor muscle position (proprioceptors). This mechanical information is converted to electrical action potentials which are then sent to the central nervous system (CNS) through its connection with proprioceptive type Ia and type II sensory neurons [1,2]. Reciprocally, after integrating the inputs from sensory neurons and those from the motor cortex through the corticospinal tract, the CNS can regulate motor activity through motoneurons (MNs): α MNs for extrafusal fibers to induce muscle contraction and γ MNs for intrafusal fibers to modulate the sensitivity of the proprioceptors. Despite their lower number in human muscle compared to extrafusal fibers [3], intrafusal fibers are indispensable for proprioception and coordination of movement. Impairment of this sensory circuit can cause motor deficits, especially in fine or coordinated motor activity [4,5].

This proprioceptive system has been extensively investigated with animal models [6,7], but research to address function in human systems has been gaining increased attention. A representative clinical problem is human deafferentation, in which a patient loses their afferent sensory input due to a number of causes, including neuropathy [8]. In the absence of proprioceptive feedback, these patients suffer difficulties in motor control. They cannot control the magnitude and speed of the motion of their limbs leading to problems in balance and gait [9]. Basic life skills such as mastication, swallowing, even walking can become difficult unless the patient exerts increased levels of mental concentration and visual monitoring [10,11]. In addition, impairment of proprioceptive sensory feedback has been found in a wide range of diseases such as autism, ADHD [12], Parkinson's disease [13], Huntington's disease, dystonia [14], neuropathy [10] and multiple sclerosis [15]. However, deficits in proprioception and motor control may only be a side effect of the disease and therefore not a primary focus in the investigation of these diseases. However, these side effects do cause significant impact to their clinical symptoms and quality of a patient's life [10,13]. Pathology of the proprioceptive function could be caused by deficits in central integration of sensory and motor systems such as in neurodegenerative/development diseases like Parkinson's and autism [13,16], or by direct damage to the intrafusal sensory system such as in deafferentation due to neuropathy which can be induced by immune system attack, viral infection, drug toxicity or other pathological conditions [8,11,15]. Developing an in vitro model of this circuit would not only provide a valuable platform for the investigation of developmental physiology, as well as function of the somatosensory-motor system, but also for the construction of relevant disease models.

Due to difficulties in translating the findings from animal models to clinical applications, human-based in vitro systems are becoming increasingly utilized for etiological studies and for drug development. The rapid expansion of the availability of stem cells in recent years has provided an avenue for the unlimited supply of human cells for tissues, and a straightforward way to generate desired cell types. In addition, induced pluripotent stem cell (iPSC) technology provides the flexibility to investigate patient genetic diversity in these in vitro models. Our goal was to develop a human-based, in vitro muscle-sensory neuron

circuit utilizing human stem cells as the source of the intrafusal fibers and sensory neurons, and to establish and characterize the resultant intrafusal innervations.

Intrafusal muscle fibers have been induced in vitro from rat embryonic muscle cells [17], and the establishment of connections with rat sensory neurons from primary DRG neurons in a defined in vitro system has previously been demonstrated [18]. In vitro induction of intrafusal fibers from human myoblasts had also been reported but by utilizing a serum-containing system and with a focus on molecular mechanism of signal transduction [19]. We have successfully differentiated functional myotubes (extrafusal fibers) from human satellite cells [20], then co-cultured them in a serum-free defined medium with motoneurons (MNs) to form NMJs [21]. We have also differentiated functional proprioceptive sensory neurons from human neural progenitors [22]. The identity of these sensory neurons has been confirmed by the expression of sensory neuron markers Brn3a and peripherin, and their proprioceptive identity has been further confirmed by the markers of parvalbumin and vGluT1 [22]. In this study, we generated intrafusal fibers from human satellite cells and established innervation by the stem cell-derived human proprioceptive sensory neurons and established functional connections. Surprisingly, the intrafusal fibers were capable of repetitively firing upon stimulation, which hadn't previously been observed in any extrafusal myofibers [20,23] or rat intrafusal myofibers [17]. This human-based, in vitro mechanosensory model could find important applications in the etiological study of relevant diseases such as deafferentiation, neuropathies and possibly neuropathic pain. It could also provide a plausible phenotypic model for the drug screening and potential therapy development for these diseases.

2. Materials and methods

2.1. DETA surface modification

Glass coverslips (6661F52, 22 × 22 mm No.1; Thomas Scientific, Swedesboro, NJ, USA) were cleaned using HCl/methanol (1:1) for a minimum of 2 h, rinsed with water, soaked in concentrated H₂SO₄ for at least 2 h and rinsed with water. The coverslips were then boiled in nanopure water and oven dried. The trimethoxysilylpropyldiethylenetri-amine (DETA, T2910KG; United Chemical Technologies Inc., Bristol, PA, USA) film was formed by the reaction of the cleaned surfaces with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (T2904; Fisher, Suwanne, GA, USA). The DETA coated coverslips were heated to ~80 °C, cooled to room temperature (RT), rinsed with toluene, reheated to approximately 80 °C, and then cured for at least 2 h at 110 °C. Surfaces were characterized by contact angle and X-ray photo-electron spectroscopy as previously described [23–25].

2.2. Induction of intrafusal fibers from human satellite cells

Human skeletal muscle stem cells (hSKM SCs)/progenitors were isolated, proliferated and differentiated as described in Thorrez et al. [26]. Briefly, the primary human skeletal muscle cells (hSKMs) were isolated by needle biopsy [27] and expanded in the myoblast growth medium (MGM; SkGM (Cambrex Bio Science, Walkersville, MD) plus 15% (v/v) fetal bovine serum. Biopsies were performed on adult volunteers according to procedures

approved by the Institutional Clinical Review Board of the Miriam Hospital. Cell preparations averaged 70% myogenic content based on desmin-positive staining [28].

To induce intrafusal fibers from these hSKM SCs, a unique protocol was developed in this study. For each culture, hSKM SCs/progenitors with about 20 doubling times were plated on DETA coverslips at a density of 100 cells/mm² in hSKM Growth Medium (Lonza, Cat# CC-3160) and fed every 2 days by changing the entire medium until confluency. Myoblast fusion was then induced by switching to the differentiation medium 1 [20] (high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with Insulin (10 µg/ml), bovine serum albumin (BSA) (50 µg/ml), Epidermal Growth Factor (10 ng/ml) and Gentamicin (50 µg/ml)). For intrafusal fiber induction, Neuregulin (10 ng/ml), Laminin (10 ng/ml) and Agrin (10 ng/ml) were added to the differentiation medium on day 0, day 4 or day 7 after the initiation of differentiation. The cells were fed every 2 days by changing half of the medium. Four days after differentiation initiation, the medium was switched to the co-culture medium or medium 2 (Table 1). The cells were fed once using the same medium 2 days later by changing half of the medium. Thereafter, the cultures were fed every 2 days using NBactive4 (Brain Bits, Nb4-500) by replacing half of the medium.

2.3. Co-culture of human sensory neurons with human intrafusal fibers

Co-cultures were established according to the procedures depicted in Fig. 1. Human sensory neurons (hSNs) were differentiated from human neural progenitor cells, STEMEZTMhNP1 (Neuromics, Edina, Minnesota) as described in Guo et al. [22]. Neuromics' product information states that their cells can be expanded through 10 passages before any genotypic monitoring is necessary (<http://www.neuromics.com/>). In this study, passage 9 or 10 cells were used. Briefly, hNP1 cells in the growth phase were manually dissociated and re-plated onto glass coverslips pre-coated with DETA, followed by Poly-ornathine/Laminin/Fibronectin [29], at a density of 400 cells/mm². The cells were expanded in the proliferation medium for 2–3 days to achieve ~90% confluence before induction. To initiate sensory neuron differentiation, the medium was replaced with KSR medium which contained 10 µM SB43152 (Tocris, Cat 1614) and 500 ng/ml Noggin (R&D, Cat 6057-NG-025). To feed the cells during differentiation, the medium was replaced and gradually switched from KSR medium (ThermoFisher, Cat 10828028) to N2B medium (NeuralStem Inc) according to the following schedule: day 0 (100% KSR, 0% NB), day 2 (75% KSR, 25% N2B), day 4 (50% KSR, 50% N2B), day 6 (25% KSR, 75% N2B), days 8 & 10 (0% KSR, 100% N2B). However, the content of SB43152 and Noggin (10 µM and 500 ng/ml, respectively) remained constant throughout the procedure. Starting with day 12, the cells were fed with a differentiation medium by changing 1/3 of the medium every 2 days. After 14 days of differentiation, SNs were harvested by trypsinization and added to the muscle culture at a density of 75 cells/mm², timed so the muscle culture was on day 4 of differentiation and right after it had been switched to co-culture medium.

2.4. Immunocytochemistry and microscopy

Cultures were analyzed with immunocytochemistry and microscopy as described previously [21,22]. Cells on the DETA cover-slips were fixed utilizing freshly prepared 4% paraformaldehyde in PBS for 15 min. Cells were then washed twice in Phosphate Buffered

Saline (PBS) (pH 7.2, w/o Mg^{2+} , Ca^{2+}) for 10 min at room temperature and then permeabilized with 0.1% triton X-100/PBS for 15 min. Non-specific binding sites were blocked using Blocking Buffer (5% Donkey serum plus 0.5% BSA in PBS) for 45 min at room temperature. Cells were then incubated with primary antibodies overnight at 4 °C. After being washed with PBS 3 × 10 min, the cells were then incubated with secondary antibodies for 2.5 h at room temperature. The cells were again washed with PBS 3 × for 10 min and mounted utilizing Vectashield with 4'-6-Diamidino-2-Phenylindole (dapi) (Vector laboratories, Inc.). Primary antibodies used in this study included: Goat-anti-Peripherin (Santa Cruz Biotech, 1:25), Rabbit-anti-MHC H-300 (Santa Cruz, 1:200), Goat-anti-PICK1 (Santa Cruz, 1:200), Mouse-anti-Neurofilament (Sigma, 1:1000), Rabbit-anti-Egr3 (Santa Cruz, 1:100), erbB2- P -Alexa488 (Millipore, 1:250). The monoclonal antibody against slow tonic muscle heavy chain (S46, 1:10) was obtained from the Developmental Studies Hybridoma Bank which is under the auspices of the NICHD and maintained by the University of Iowa. Phalloidin-568 (Life Technologies, 1:40) was included during the secondary antibody incubation. The monoclonal antibody against cardiac muscle heavy chain BA-G5 was produced as described in Rumsey et al. [17]. Secondary antibodies include: Donkey-anti-Mouse-568 (Invitrogen, 1:250), Donkey-anti-Rabbit-488 (Invitrogen, 1:250), and Donkey-anti-Rabbit-568 (Invitrogen, 1:250) Donkey-anti-Gt-488 (Invitrogen, 1:250). All antibodies were diluted in Blocking Buffer.

2.5. Electrophysiological recording

Electrophysiological properties of human intrafusal fibers were investigated after ~10 days of co-culture with human sensory neurons utilizing whole-cell patch-clamp recording techniques [25]. The recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope [30].

Intrafusal fibers were identified visually under an infrared DIC-video microscope by their typical bag fiber morphology. Patch pipettes, with a resistance of 6–10 M Ω , were made from borosilicate glass (BF 150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller (Sutter Instrument Company). Current-clamp and voltage-clamp recordings were made utilizing a Multiclamp 700A amplifier (Axon, Union City, CA). According to the standard protocol that has been used routinely [25,31,32], the pipette (intracellular) solution contained (in mM) K-gluconate 140, $MgCl_2$ 2, Na_2ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg and Hepes 10; pH 7.2. After the formation of a giga ohm seal and membrane puncture, the cell capacitance was compensated. The series resistance was typically <23 M Ω , and it was compensated 60% using the amplifier circuitry. Signals were filtered at 3 kHz and sampled at 20 k Hz using a Digidata 1322A interface (Axon instrument). Data recording and analysis were performed with pClamp8 software (Axon instrument). Membrane potentials were corrected by the subtraction of a 15 mV tip potential, which is the liquid junction potential between intracellular solution and extracellular solution and was calculated using Axon's pClamp8 program, and it was within the normal range of junction potential for patch clamp (10–20 mV) [33]. Membrane resistance and capacitance were calculated using 50 ms voltage steps from –85 to –95 mV without any whole-cell or series resistance compensation. The resting membrane potential and depolarization-evoked action potentials were recorded in current-clamp mode.

Depolarization-evoked inward and outward currents were examined in voltage-clamp mode. Single APs were elicited by a brief saturated depolarization current.

2.6. Quantification

For the quantification of bag fiber induction under phase microscopy, a minimum of 10 random fields were imaged under phase microscopy. The number of bag fibers out of the total number of myotubes was quantified for each experimental condition and compared to the control. At least 3 independent cultures were analyzed for each condition. Statistical analysis by T-Test (one tail, two samples with unequal variance) was performed to compare differences between samples and $P < 0.05$ was considered significantly different.

3. Results

3.1. Development of a defined system for the induction of intrafusal fibers, utilizing a serum-free medium

Considering the common origin, environment and multiple needs shared by both intrafusal and extrafusal fibers during development, the protocol for generating functional extrafusal fibers [17,18] developed in our laboratory was used as a starting point for designing protocols for intrafusal fiber induction. As previously reported, neuregulin 1- β -1 (NRG) is necessary for the specification of nuclear bag fibers and for spindle development [19,34]. Additionally, the basal laminar molecules, laminin and agrin, are especially important for NRG signaling for intrafusal induction by activating a dystroglycan receptor on the myotube membrane which then potentiates the NRG-Egr3 signaling pathway [35]. Therefore, these three factors, NRG, laminin and agrin, were included in the differentiation process. The composition of medium 2 is as described in Table 1.

3.2. Phase contrast evaluation of the induction of intrafusal fibers

The human satellite cells were plated, expanded and differentiated as described in the Methods. Myocyte fusion was initiated after switching to medium 1, which defines the start of the differentiation process, and multinuclear myocytes were evident after 3–4 days in vitro. The cultures are then switched to medium 2 and multinuclear myotubes continued to mature as indicated by more individualized myotubes with better defined morphology under phase microscopy. Starting approximately at day 7, myofibers with morphology consistent with bag fibers could be identified, and were increasingly evident and numerous as the culture matured. Some demonstrated equatorial nucleation which is typical for bag fibers [1,36] (Fig. 2 A, C). Myofibers with a chain of nuclei were also observed frequently and identified as chain fibers (Fig. 2B).

During development, nuclear bag morphology is thought to be the earliest reliable feature for identifying young intrafusal fiber bundles in human muscle [36], however, the identification of chain fibers under phase microscopy is not as well defined. Thus, the number of induced muscle bag fibers was used to optimize the induction protocol. To determine whether the generation of bag fibers was NRG-dependent as postulated from in vivo studies [37], the percentage of bag fibers over the total number of myofibers was compared with control cultures in which the three factors were omitted (NRG, laminin and

agrin). Also, to determine the optimal timing of factor addition, the percentage of bag fibers was analyzed from factors inclusive from day 0, 4 & 7 of differentiation in medium 1. As displayed in Fig. 2D, inclusion of the three factors caused a significant increase of the percentage of bag fibers from $3.02 \pm 3.95\%$ to at least $15.80 \pm 6.62\%$ ($P < 0.05$). But there is no significant difference when added at different time points during differentiation in medium 1. Later analysis indicated that switching to medium 2 on day 4 generated more consistent results in intrafusal induction than on day 7. Therefore, cells were induced for 4 days in medium 1 before switching to medium 2 in all subsequent experiments.

3.3. Immunocytochemical evaluation of intrafusal fiber induction

Immunostaining was used to confirm the identity of the induced bag fibers. The hSKM culture treated with NRG/LMN/Agrin as described above, was evaluated for the expression of the bag fiber-specific myosin heavy chain (MHC) with the S46 antibody [38,39]. As indicated in Fig. 3A, a sub-population of myofibers expressed the slow tonic MHC and were identified as S46-positive myotubes. In addition, these bag fibers expressed α cardiac-like MHC which was indicated by immunocytochemical staining with the BA-G5 antibody (Fig. 3C) [38,40]. In vivo, NRG induction functions through the ErbB2 receptor [41]. Its activation through phosphorylation can then increase the expression of the transcription factor Egr3 [37], which triggers downstream genes that then delineate intrafusal fiber differentiation. Therefore, the induced cultures were analyzed for the expression and distribution of phosphorylated ErbB2 (ErbB2- $\textcircled{P}$) and Egr3 (Fig. 4). ErbB2- $\textcircled{P}$ was specific for a subset of myofibers and was found in all S46-positive myotubes (Fig. 4A). The induced cultures were then analyzed for the expression of Egr3, the intrafusal fiber-specific transcription factor. As in Fig. 4B&C, Egr3 was found to be expressed in bag fibers which were also identified by staining for the S46-antibody. Both the expression of ErbB2- $\textcircled{P}$ and Egr3 in the induced intrafusal fibers suggest the activation of the ErbB2 receptor-mediated signaling pathway by NRG treatment, similar to what occurs during in vivo development.

3.4. Immunocytochemical evaluation of sensory nerve endings on intrafusal fibers

The induced intrafusal fibers were co-cultured with human stem cell-derived sensory neurons [22] and evaluated for innervation characteristic of this mechanosensory system. The differentiated sensory neurons were added to the partially differentiated muscle culture (after step 1 differentiation) and co-cultured for approximately 10 days (Fig. 1). Both sensory neurons and intrafusal fibers survived well in the co-culture system as indicated by the phase images (Fig. 5A). In order to evaluate the synaptic connections the co-cultures were immunostained with the sensory neuron marker peripherin and the intrafusal marker BA-G5. In vivo sensory nerve endings on intrafusal fibers normally demonstrate two distinct morphological structures: annulospiral wrappings (ASWs) and flower spray endings (FSEs) [1]. As shown in Fig. 5 (B–C), both terminal structures were observed in the developed co-culture system. An example of the annulospiral association between sensory terminals and the intrafusal fibers is demonstrated in the detailed optical series depicted in Fig. 6.

3.5. Electrophysiological properties of intrafusal fibers in the co-culture

The electrophysiological properties of the intrafusal fibers in the co-culture were evaluated using voltage and current clamp recordings. Representative voltage-clamp and current-clamp

recordings for intrafusal fibers are shown in Fig. 7. Similar to extrafusal fibers [20,42], intrafusal fibers demonstrated prominent sodium and potassium currents and generated action potentials. Most interestingly, repetitive firing was observed in 10 out of 19 bag fibers recorded in the system (Fig. 7B). The electrophysiological properties of intrafusal fibers are listed in Table 2.

3.6. Immunocytochemical analysis indicates innervation of intrafusal fibers by human sensory neurons

To examine in detail the connections between the sensory neuron terminals and intrafusal fibers, the co-culture was analyzed by immunostaining with PICK1 (PRKCA-binding protein) and peripherin. PICK1 contains a PDZ domain and functions as an adaptor protein that organizes a variety of membrane proteins including the stretch sensitive sodium channel BNaC1 on the membrane of intrafusal fibers. Co-localization of PICK1 with nerve terminals was observed. Fig. 8 demonstrates an example of a flower spray endings associated with a myofiber and its co-localization with PICK1, suggesting a functional connection between intrafusal fibers with the axonal terminals of sensory neurons, which is a key connection for mechanosensory function.

4. Discussion

This study reports the de novo induction of human intrafusal skeletal muscle fibers from satellite cells and their synaptic connection with human stem cell-derived sensory neurons in a defined in vitro system. The induction of intrafusal fibers was characterized by morphology and fiber-type specific transcription factor expression using phase microscopy and immunocytochemistry. The connections between sensory neurons and the intrafusal fibers were observed morphologically by type-specific innervation patterns using phase microscopy and immunocytochemistry. The functional maturity of the induced intrafusal fibers was confirmed by patch-clamp electrophysiological analysis.

During development, induction of muscle spindles depends on trophic factors released by sensory neurons. Neuregulin (NRG), specifically the Ig-Nrg1 isoform, is a proteoglycan released by type Ia proprioceptive sensory neurons and is sufficient to induce muscle spindle differentiation in vivo [37]. The NRG-ErbB2-Egr3 signaling pathway has been proposed to be the central pathway for this effect [2]. Mice with a conditional knockout of ErbB2 have progressive defects in proprioception due to the loss of muscle spindles [4]. Egr3 has an essential role for the phenotypic differentiation of spindles [43] and mice deficient in Egr3 have gait ataxia and lack muscle spindles [44]. Therefore, it is hypothesized that NRG released by sensory axons, through the activation of ErbB2 receptors on the myotube membrane, can turn on the transcription factors Egr3/Pea3/Erm, which then initiates downstream genes and delineates the differentiation of intrafusal fibers and muscle spindles [2,4,19,34,37,43,44]. In addition to this major pathway, the basal laminar molecules, laminin and agrin, are especially important for NRG signaling and intrafusal induction by activating a dystroglycan receptor on the myotube membrane which then potentiates the NRG-Egr3 signaling pathway [35]. In this study, both pathways were employed for the in vitro

induction of intrafusal myofibers as evidenced by the increased expression of ErbB2- P and Egr3 in BA-G5-positive myotubes.

The electrical function of the induced intrafusal fibers was confirmed by patch-clamp electrophysiological analysis. However, these results differed from previous experiments with human extrafusal fibers, which only fire a single action potential during depolarization [20], in that the majority (53%) of the intrafusal fibers examined exhibited repetitive firing. The rest fired single action potentials probably due to maturation issues. This repetitive firing phenomenon has not been observed in the electrophysiology of rat intrafusal fibers [17]. This is the first electrophysiological analysis of human intrafusal fibers and the first report concerning the electrophysiological property of these myotubes. This unique electrophysiological result for a human muscle fiber may actually be intrinsic to its function as a mechano-electrical transducer. Sensory receptors of multiple modalities utilize firing frequency to encode stimulus intensity such as the sensing of temperature, pain and touch [48–52]. Thus, repetitive firing may be important for the normal function of these sensory transducers, endowing them the potential to code stimulus intensity with firing frequency. This result is in agreement with numerous previous studies from human microneurography, an in vivo approach to monitor the impulses in sensory nerves while applying the stimulus, such as touch or movement [52,53]. All the dynamic responses of human muscle intrafusal sensory afferents from these studies displayed multiple discharges at various frequencies depending on the stimulation protocols [45–47,52]. Overall, these results establish that there are important electrophysiological differences between intrafusal and extrafusal fibers, and also between human intrafusal and rat intrafusal fibers, which could have new implications for understanding muscle physiology.

In vitro systems composed of the main component of the specialized stretch receptors could allow studies of perception and coordination of limb movement in a defined, controlled system. Mice lacking spindle fibers within skeletal muscle present with profound gait ataxia and other motor deficits [44]. Impairment of proprioception has been well represented in deafferentation cases in which proprioceptive sensory nerves are damaged due to neuropathy [8,54], and can also be associated with many diseases including multiple sclerosis [55], spinal cord injury [56], diabetes mellitus [57] and other degenerative diseases [13]. Humans with proprioception dysfunction typically have deficits in motor planning, motor control, grading movement and postural stability. While previous publications covering motor control/learning as well as rehabilitation have been obtained from deafferented patients [58–60], a human-based in vitro system would constitute a valuable phenotypic model for this sensorimotor circuit. The in vitro system reported in this study using a well-defined serum-free medium provides a platform for the dissection of the physiological/molecular/developmental mechanism and regulation of this mechanosensory system. The human nature of this biological system makes it more relevant and amenable to the study of neurological and/or muscular disease modeling, drug discovery and regenerative medicine.

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Fig. 1.
Schematic diagram of the culture protocol and timeline.

Fig. 2. Phase contrast micrographs. A) A bag fiber in an induced culture after 16 days of differentiation. Note the equatorial distribution of multiple nuclei in the bag-shaped myofiber. Scale bar: 20 μ m. B) A chain fiber in an induced culture after 16 days of differentiation. Note the linear assembly of the nuclei inside the myotube. Scale bar: 20 μ m. C) A low magnification image of an induced culture after 17 days of differentiation. Scale bar: 80 μ m. D) Graph comparison of the percentage of bag fibers when NRG/LMN/FN was added to the culture at different times during differentiation (* means $P < 0.05$).

Fig. 3.

A&B) Immunocytochemistry of induced intrafusal muscle cultures stained with the bag fiber-specific antibody S46 (green), and co-stained with Phalloidin (red) which is a general marker for all myofibers. A) A representative bag fiber is highlighted with a yellow arrow and a blue arrow indicates a myofiber that was partially stained by S46 but did not present apparent bag morphology, suggests the possibility of a chain fiber or an emerging bag fiber formation. B) An image of a bag-fiber at higher magnification. C) A bag fiber immunostained with the BA-G5 antibody.

Fig. 4.

Activation of Neuregulin signaling pathway demonstrated by immunocytochemistry. A) Co-immunostaining of Phalloidin and erbB2- P . To visualize the erbB2- P clusters on the cell membrane, two regions of the low magnification image were enlarged. Image a' and b' are the higher magnifications of regions a and b respectively. Abundant erbB2- P signals (indicated by arrows) were observed only on multi-nuclei bag fibers (b and b'), and rarely observed on the others (a and a'). B) Immunostaining of Egr3 co-stained with S46. Egr3-positivity was only observed in S46-positive myofibers, confirming its specificity. C) A bag fiber under higher magnification.

Fig. 5. Immunocytochemical analysis of the co-culture of human sensory neurons and intrafusal fibers indicated connectivity. A) Phase image of a Day 10 co-culture. Note the pseudo-unipolar sensory neuron (orange arrow) and bipolar sensory neuron (red arrow) in the vicinity of a bag-shaped myofiber. B&C) Co-immunostaining of Peripherin and BA-G5 revealed two typical sensory terminal structures around intrafusal fibers: annulospiral wrappings (B) and flower spray endings (C), as indicated by the arrows in both images.

Fig. 6. Optical section of a sensory terminal structure indicating annulospiral wrappings on intrafusal fibers immunostained with Peripherin and BA-G5. A) Projected image of an intrafusal fiber with sensory axons. B) Series of optical sections of the intrafusal fiber demonstrated in A).

Fig. 7. Patch clamp recording from intrafusal fibers in the co-culture system. A) Phase micrograph of the recorded cell. B) Current clamp recording indicating repetitive firing of APs. C) An example trace of active Na^+ and K^+ currents from a voltage clamp recording. D) An example trace of an Action Potential (AP) elicited when the cell received a saturated stimulus (2 msec 200 pA inward current).

Fig. 8.

Immunocytochemical analysis of the connection of human sensory neurons and intrafusal fibers from a day 5 co-culture by co-immunostaining with PICK1 and Neuro-filament antibodies. Co-localization of PICK1 expression on the myofiber at the neural terminal ending site is indicated by the arrow.

Table 1

Composition of enriched Co-culture media (medium 2).

Component	Full name	Concentration	Company	Catalog number
Neurobasal/Neurobasal A			Invitrogen	10888/21103
B27 (50X)		1X	Invitrogen	17504-044
Glutamax (100X)		1X	Invitrogen	35050
rhNRG	Neuregulin-1- β 1 EGF domain	100 ng/ml	R&D	396-GF/CF
Rh β -NGF	Nerve Growth Factor	100 ng/ml	R&D	256-GF/CF
GDNF	Glial-derived Neurotrophic Factor	10 ng/ml	Cell Sciences	CRG400B
BDNF	Brain-derived Neurotrophic Factor	20 ng/ml	Cell Sciences	CRB600B
Shh	Sonic Hedgehog, N-terminal peptide	50 ng/ml	R&D	1845-SH-025
RA	Retinoic Acid	0.1 uM	Sigma	R2625
IGF-1	Insulin-like Growth Factor -I	10 ng/ml	PeproTech	100-11
cAMP	Adenosine 3',5'-cyclic Monophosphate	1 uM	Sigma	A9501
CNTF	Ciliary Neurotrophic Factor	5 ng/ml	Cell Sciences	CRC400A
NT-3	Neurotrophin-3	20 ng/ml	Cell Sciences	CRN500B
NT-4	Neurotrophin-4	20 ng/ml	Cell Sciences	CRN501B
Vitronectin		100 ng/ml	Sigma	V8379
Laminin	Mouse Laminin	4 μ g/ml	Invitrogen	23017-015
G5 (100X)		1X	Invitrogen	17503-012
Agrin		100 ng/ml	R&D	550-AG-100

Electrophysiological properties of induced intrafusal bag fibers in the co-culture with human sensory neurons.

Table 2

	Resting membrane potential (mV)	Membrane resistance (M Ω)	Membrane capacitance (pF)	No. of AP (s)	Inward current (pA)	Outward current (pA)	AP amplitude (mV)
Average $\pm$ STDEV	-53.0 ± 9.6	365.2 ± 235.9	207.9 ± 65.0	3.1 ± 2.5	5043.6 ± 1888.8	1932.3 ± 1311.9	95.9 ± 11.5