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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.

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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 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 Υ 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

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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 H_2SO_4 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 photoelectron 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/mm^2 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, STEMEZ^{TM}hNP1 (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/mm^2. The cells were expanded in the

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