Review

Generating intrafusal skeletal muscle fibres *in vitro*: Current state of the art and future challenges

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Abstract

Intrafusal fibres are a specialised cell population in skeletal muscle, found within the muscle spindle. These fibres have a mechano-sensory capacity, forming part of the monosynaptic stretch-reflex arc, a key component responsible for proprioceptive function. Impairment of proprioception and associated dysfunction of the muscle spindle is linked with many neuromuscular diseases. Research to-date has largely been undertaken *in vivo* or using *ex vivo* preparations. These studies have provided a foundation for our understanding of muscle spindle physiology, however, the cellular and molecular mechanisms which underpin physiological changes are yet to be fully elucidated. Therefrom, the use of *in vitro* models has been proposed, whereby intrafusal fibres can be generated *de novo*. Although there has been progress, it is predominantly a developing and evolving area of research. This narrative review presents the current state of art in this area and proposes the direction of future work, with the aim of providing novel pre-clinical and clinical applications.

Keywords

Skeletal muscle, muscle spindle, intrafusal fibre, proprioception, tissue engineering

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Introduction

Proprioception is an integrated, multi-system physiological function, which can be described as the 'sense of position and movement of a part of the body, relative to another part'. Proprioception is imperative to coordinated movements, body posture, balance, postural control and influencing motor learning and relearning.[1] Such a function is achieved through a somatosensory input – feedback loop necessary to determine the change and extent of muscle length, tension (passive and active force), consequential changes in joint angle and associated movement of the skin.[2] Mechanoreceptors situated in the joint, including the joint capsule, ligaments, muscles, tendons and skin all contribute to proprioceptive function.[3–5] However, current knowledge indicates that the muscle spindle (MS) provides the greatest contribution to proprioceptive function.[6]

This review will provide a brief overview and summary of the anatomy, physiology and function of the muscle spindle, as well as introducing clinical conditions where muscle spindle dysfunction is observed. The main aim is to summarise how our understanding of muscle spindle morphogenesis, has informed the development of *in vitro* models of intrafusal fibres. The development and progress of these models will be discussed, with the challenges and future directions related to the development of physiological and biomimetic *in vitro* models of the muscle spindle.

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Anatomy and physiology of the muscle spindle

The MS is described as a mechano-sensory organ, that detects and mediates static and dynamic information about skeletal muscle fibre length and stretch. Sensory information is communicated to the central nervous system, where it mediates the appropriate motor response.[7] Muscle spindles are present within most skeletal muscles and are at a higher density in muscles responsible for fine motor control or postural adjustment, such as the hand, head or neck.[8,9] They are embedded within skeletal muscle, running parallel and surrounded by regular fascicles of force producing fibres (extrafusal fibres). They consist of an encapsulated bundle of intrafusal muscle fibres within a distinct extra-cellular matrix (ECM) capsule, innervated by primary (Ia) and secondary (II) afferent sensory neurons, as well as dynamic and static efferent γ-motor neurons, termed fusimotors[10,11] (Figure 1).

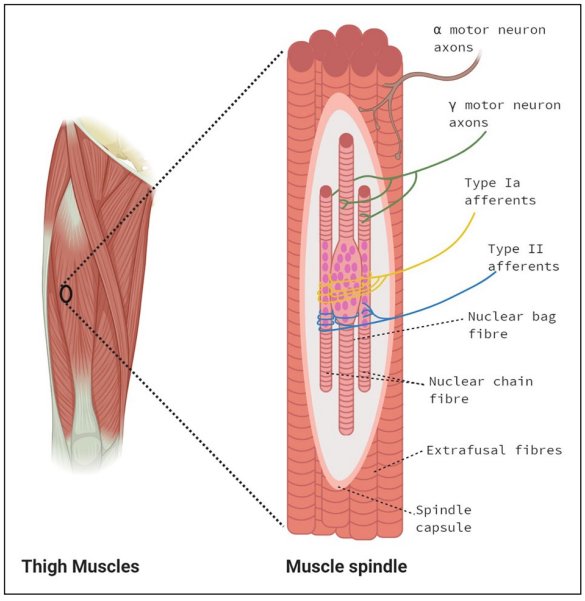

Figure 1. Simplified diagram of the anatomical structure of the muscle spindle. The muscle spindle consists of an encapsulated bundle of intrafusal 'bag' and 'chain' fibres, which run parallel with normal force producing fibres (extrafusal). The muscle spindle consists of a non-contractile equatorial region (smooth) and contractile (ribbed) polar regions. Type I afferents innervate the equatorial regions of with ASWs. The Type II afferents innervate the juxta equatorial regions with both annulospiral wrappings (ASWs) and flower spray endings (FSEs). In the contractile polar regions, intrafusal fibres are innervated by either static or dynamic gamma motor neurons which modulate the sensitivity of the afferent fibres through contraction of the polar regions. Myonuclei in the bag fibres are clustered at the equatorial regions with a 'bulging', appearance. Myonuclei in the chain fibres are aligned linearly across the equatorial regions. Figure created with BioRender.com.

There are three sub-types of intrafusal fibres; nuclear bag_1, bag_2 and chain, where there are commonly 2–3 bag fibres and 4–6 chain fibres per MS.[12] To date, these fibres have been categorised based on morphological criteria, myosin ATPase immunohistochemical staining profile, innervation pattern, functional responses and expression of specific myosin heavy chains.[6,13] The nuclei of bag_1 and bag_2 fibres cluster in the equatorial region, creating a bulging 'bag like' appearance. The nuclei of chain fibres align linearly, much like extrafusal fibres.[10] The innervation pattern of the sub-types of intrafusal fibres is also specialised according to physiological function. In mammals, type Ia afferent innervation occurs at the equatorial region of all intrafusal fibre types, forming specialised synapses called annulospiral wrappings (ASWs). Type IIa afferents primarily originate at the juxta-equatorial regions and polar regions of nuclear chain fibres, and less frequently bag_2 and bag_1 bag fibres,[14,15] forming either unique flower spray endings (FSEs) or ASWs similar to Ia afferent endings.[6,15,16] Investigation of the human MS largely corroborates with mammalian data.[17] Fusimotor activity modulates the sensitivity of muscle spindle afferents through contraction of the polar regions of intrafusal fibres.[11,12] It is generally accepted that the dynamic fusimotors only innervate bag_1 fibres, while the static fusimotors innervate bag_2 and chain fibres.[10] The structural and molecular differences suggest independent functions for each intrafusal fibre type. The number and size of intrafusal fibres, their typing as determined by myosin ATPase staining and MyHC expression is highly heterogeneous between spindles from different human muscles.[13,18,19] For example, biceps brachii muscle spindles have a smaller mean intrafusal fibre diameter and proportionally contain significantly less bag_2 and chain fibres than spindles from masseter muscle.[18] This highlights the complexity of MS physiology and indicates functional specialisation among different muscles of the same species.

Describing the extensive and complex physiology of the muscle spindle is beyond the scope of this specific review, however to gain further insight into the MS we recommend comprehensive review articles.[6,11–13]

Clinical significance

Impairment of proprioception and associated dysfunction of the MS is linked with many neuromuscular diseases including; multiple sclerosis[20–22] Parkinson's disease,[23,24] muscular dystrophy,[25] and peripheral nerve injuries.[22,26–30] Proprioception also deteriorates with age, contributing to overall musculoskeletal frailty and morbidity.[31,32] There is a body of literature to support the impairment and decline of proprioception in diabetes,[33–35] thought to be related to degeneration of both the muscle spindle and neurons in the hyperglycaemic/hyperinsulinaemic state. Patients with

proprioceptive dysfunction display a variety of difficulties in controlling the speed and magnitude of limb movement, which affects basic motor skills and causes significant physical limitations, such as impaired balance, locomotion and postural stability.[34,36–39]

To date, much of the research has been focussed on the cellular mechanisms regulating the effector motor function of skeletal muscle tissue. This work, although highly significant, often neglects the integrated nature of the neuromuscular system by not accounting for the afferent functions of skeletal muscle, mediated via the muscle spindle. A focus in both basic science and clinical studies towards improving complete neuromuscular function following dysfunction, is likely to be more effective in providing the development of novel therapeutic and prognostic strategies to enhance patient care. To this end, the development of robust models, including intrafusal fibres, are needed.

Development of intrafusal fibres and the muscle spindle

The development and maturation of the MS to full physiological function takes considerable time, during gestation and postnatally. In the rat hind limbs, the MS matures by the fourth week post birth.[40] However, it is possible to identify intrafusal fibre specification (based on myosin heavy chain (MyHC) expression) much earlier in the perinatal period.[40] Intrafusal fibres develop from primary myotubes, which are formed from the fusion of committed myoblasts during myogenesis.[41] During foetal developmental stages, as early as day E14.5 in mice, neuronal input is established and correlates with the onset of myogenic differentiation.[42] Sensory neuron Ia-afferent innervation, provides the foremost driving signal for MS morphogenesis. Ia afferent contact with the developing myotubes initiates intrafusal differentiation, through a mechanism involving the transcriptional regulator early growth response protein-3 (Egr3).[43–46] When rats are surgically denervated[47] or treated with a neurotoxin (β bungarotoxin)[48] during prenatal myogenic differentiation, MS do not develop. Surgical manipulation of sensory input at early postnatal stages in the rat, also causes fast-onset MS degeneration,[49,50] whereas ablation of motor input fails to cause any noticeable effect in early MS differentiation.[51] As such, this confirms the indispensable requirement of the sensory input for intrafusal fibre development and spindle morphogenesis.

Egr3 is a downstream target of tyrosine kinase receptors ErbB(2–4) (erythroblastic leukemia viral oncogene homologue), which are activated through neuregulin-1 (Nrg-1), a cell adhesion molecule secreted by the afferent neurons.[52] Disruption of these key signalling mechanisms, results in impairments in MS development and function. Indeed, Nrg-1 knockout mice exhibit an early defect in MS differentiation[52] and deletion of ErbB2 in mice prevents the complete formation of the MS, leading to significant neuromuscular defects, such as abnormal gait and posture.[53,54] In addition, skeletal muscle specific ablation of Egr3 results in perturbed spindle development, Ia-afferent contacted myotubes developed Egr3 negative spindle remnants with significant physiological abnormalities which persisted into adulthood.[45] As the key developmental mechanisms have been described (Figure 2), this has provided an opportunity to target these proteins when seeking to generate intrafusal fibres *in vitro*. To this end, the use of Nrg-1 has been largely reported in published work to-date.[55–61]

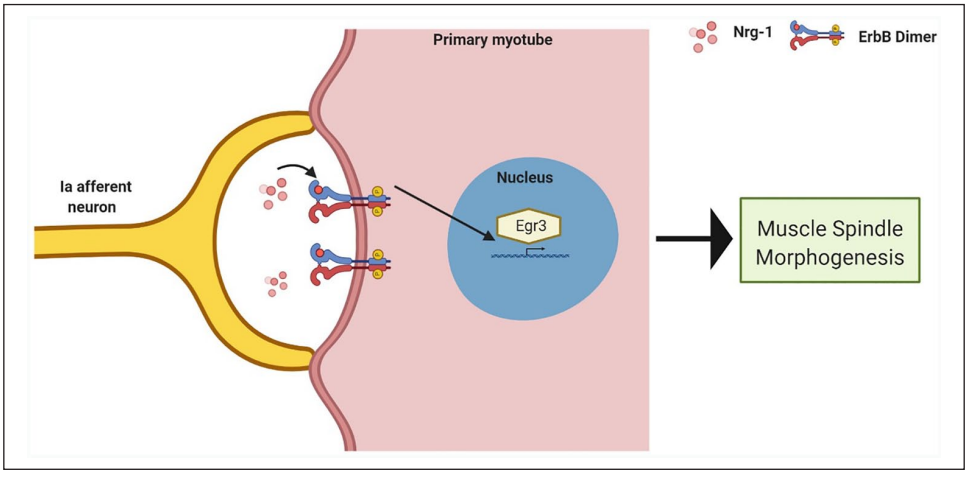

Figure 2. Proposed developmental signalling mechanism which drives intrafusal fibre differentiation and muscle spindle morphogenesis. Nrg-1 is released by an innervating Ia afferent neuron, this activates the ErbB receptors on primary myotubes. Egr3 is activated downstream of activated ErbB receptors and is essential for intrafusal formation and muscle spindle morphogenesis.
Figure created with BioRender.com.

Regenerative capability of the muscle spindle

Post-natal muscle regeneration recapitulates many of the cellular and molecular aspects of muscle embryonic development.[62] Satellite cells are the resident stem cell of skeletal muscle, where they reside beneath the basal lamina surrounding each muscle fibre. When activated, they give rise to committed myoblasts which are essential for muscle fibre regeneration and repair.[63,64] There is growing evidence to suggest that intrafusal fibres are in a comparatively immature state, relative to mature extrafusal fibres. In chickens, there is a higher number of satellite cells residing within intrafusal fibres of the MS. These cells also maintain expression of paired-domain transcription factor 3 (PAX3), a satellite cell marker which is repressed in the majority of mouse muscles following embryogenesis.[65–67] Furthermore, myonuclei of intrafusal fibres maintain gene expression of Myf5,[68] the earliest marker of myogenic commitment in satellite cells, and retain expression of embryonic and neonatal isoforms of MyHC into maturity.[69,70] Finally, intrafusal fibre growth is arrested shortly after birth, where they remain comparatively small compared to extrafusal fibres.[71] Together, these data suggest a unique cellular and molecular phenotype of intrafusal fibres. The physiological advantage of increased satellite cell number and maintained expression of numerous factors associated with early muscle development in MS, is currently unknown. It has led conjecture that due to the continuous length monitoring of skeletal muscle and adjustment of its own length, intrafusal fibres may require regular regeneration. Further, that by remaining in this comparatively immature state contributes to a greater capacity for intrafusal fibre regeneration, repair and preservation.[66] Recent work supports this hypothesis, whereby adult mice ablated for satellite cells displayed normal adaptability to aerobic exercise but showed decrements in wheel running performance and gross motor coordination.[72] This is a similar phenotype seen in universal or skeletal muscle specific Egr3 knockout mice, which lack functional MS.[45,46,73] Upon immunohistochemical analysis, the MS exhibited an increased ECM deposition and presented intrafusal fibre atrophy, while extrafusal fibres displayed no abnormal morphology. In addition, satellite cell depletion lead to functional changes in MS, as determined by decreased firing frequency in response to stretch. Combined, these data indicate satellite cells are essential for MS maintenance and regeneration and that there is a key physiological demand for the relative increased satellite cell density in intrafusal fibres.

Despite this evidence, the specific cellular and molecular mechanisms which regulate regeneration of the MS are yet to be fully elucidated. This highlights a necessity to develop robust and tractable models, to investigate how muscle spindle regeneration is controlled and affected in health and disease.

Generating intrafusal fibres *in vitro*

Due to the complex integrated physiology of the MS, *in vivo* experimentation largely conducted in mice, rats and cats has formed the basis of much of our current understanding.[6,10,11] *In vivo* and *ex vivo* experiments maintain the muscle spindle within the majority of its the physiological niche with functional innervation. Additionally, there are powerful genetic tools, including transgenic animals and optogenetic tools available to induce various physicochemical and cellular alterations, as well as models of injury and disease states.[74]

There are however limitations of *in vivo* and *ex vivo* models. Invasive experimentation on mammalian models are ethically undesirable. The 3Rs – Replacement, Reduction and Refinement – are embedded into the legislation and guidelines governing the ethics of animal use in experiments, and highlights that, if possible, methods which avoid or replace the use of animals should be developed.[75] Analytical techniques used in animal studies often cannot be safely reproduced in humans, experiments are expensive, time consuming and yield data which may not accurately translate to human physiology.[76,77] Furthermore, single factor changes are difficult to study due to the biological complexity of whole-body living systems and organisms.[78,79] *In vitro* modelling is an indispensable tool in biological research and in recent years there has been a shift from flat, two-dimensional (2D) cultures, to three-dimensional (3D) structures which can be manipulated to replicate a more *in vivo*-like biochemical and biomechanical microenvironment.[80] With this in mind, there is a growing need to develop robust *in vitro* models of intrafusal fibre development and function, which consider the *in vivo* anatomy and physiology described. This will help to investigate the basic molecular and cellular mechanisms regulating their phenotype, in a defined, highly controlled, reproducible system. Furthermore, there are potential pre-clinical and clinical applications for intrafusal fibres generated *in vitro*, which may contribute to the development of novel therapeutic strategies for tissue damage and disease.

In 2004, Jacobson and colleagues used recombinant Nrg-1(NRG-1) to induce the expression of Egr3 at an mRNA and protein level in a 2D human primary myoblast culture.[56] NRG-1 treated cells showed a significant increase in the expression of muscle spindle-specific slow developmental myosin, replicating the characterised developmental mechanism *in vitro*. More recent work completed by the Hickman laboratory since 2008, used NRG-1 to influence myotube fate specification towards intrafusal-like myotubes in human and rat myoblasts, or human induced pluripotent stem cells (hiPSCs).[57–61] Immunocytochemistry for intrafusal specific protein MyHC6, Egr3, phosphor-ErbB2 and distinctive morphology allowed identification of intrafusal bag fibres in a heterogenous myotube population (Figure 3). In this regard, NRG-1 induced a five-fold increase in bag-like myotubes, which corroborates with *in vivo* data

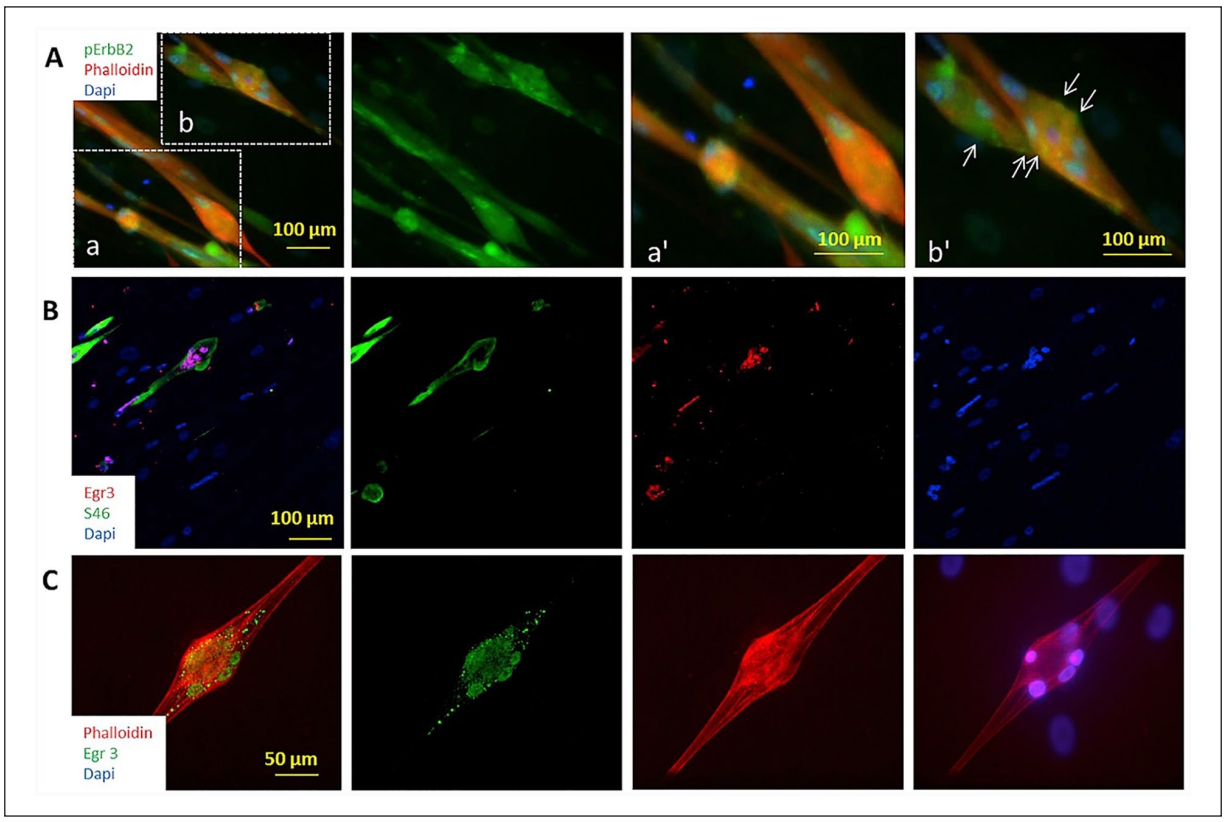

Figure 3. Activation of Neuregulin signalling pathway demonstrated by immunocytochemistry. (A) Co-immunostaining of Phalloidin and erbB2-Ⓟ. To visualise the erbB2-Ⓟ clusters on the cell membrane, two regions of the low magnification image were enlarged. Image a' and b' are the higher magnifications of regions a and b respectively. Abundant erbB2-Ⓟ signals (indicated by arrows) were observed only on multi-nuclei bag fibres (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 myofibres, confirming its specificity. (C) A bag fibre under higher magnification showing Egr3 staining.
Source: Figure from Guo[59] with permission from Elsevier.

highlighting a requirement for Nrg-1 for physiological intrafusal fibre differentiation. In addition, bag-like myotubes (MyHC6 positive) had an increased expression of phospho-ErbB2 and Egr3, indicating the developmental mechanism essential for spindle morphogenesis (Figure 2) has been activated in these cells.[59,61] This work provides evidence for the morphological, mechanistic and phenotypic (myosin heavy chain expression) development of intrafusal-like fibres *in vitro*. The morphological and phenotypic characteristics that can be used to determine the formation of intrafusal fibres *in vitro* are presented below (Figure 3 and Table 1).

S46 and BA-G5 myosin heavy chain antibodies against MyHC6, are reported to be the most intrafusal-bag fibre specific markers available.[69,82,83] In addition, MyHC15 and MyHC7b/14 have displayed specificity to specialised muscle, including intrafusal fibres.[84,85] To accurately model the extent of *de novo* intrafusal fibre formation *in vitro*, it is necessary to characterise intrafusal nuclear chain fibres separate from morphologically similar extrafusal fibres in a heterogeneous myotube population. The combination of Egr3 and phosphor-ErbB2 positive staining has been used to identify both intrafusal bag and chain fibres, however in some published manuscripts where intrafusal fibres are quantified, nuclear chain fibres have not been considered.[57–59] Intrafusal chain fibres were considered in a co-culture of dorsal root ganglion (DRG) explants and skeletal muscle cells, classification relied on an overlay in fluorescent staining for neurofilament 200, α-actin and DAPI, visualising sensory neurons and myotubes respectively, suggesting innervation[55] (Figure 4). The supplementation of NRG-1 into the co-culture system lead to increased neuronal outgrowth and migration from DRG explants and increased level of intrafusal bag and chain myotubes.

Defining bag_1 from bag_2 fibres presents another challenge in vitro; bag_1 fibres, compared to bag_2 fibres have a slower phenotype, distinguishable myosin ATPase reactivity and the of lack key M-band protein expression.[13] The in vivo assays have not yet been adapted or utilised in vitro to define bag_1 from bag_2 fibres. Alongside this, MyHC isoforms are very similar, meaning there is possible limitations caused by cross-reactivity of antibodies.[13] MyHC

Table 1. Methods proposed for the characterisation of intrafusal fibres *in vitro*.

Parameter	Description	Reference(s)
Morphological structure	Clustering of nuclei and expanded equatorial region of the myotube	Rumsey et al.[57]
Expression of developmental proteins	Expression of phospho-ErbB2 and Egr3	Rumsey et al.,[57] Colón et al.,[58,61] and Guo et al.[59]
Expression of distinctive phenotypic proteins	Expression of MyHC6	Rumsey et al.,[57] Colón et al.,[58] and Guo et al.[59,81]
Expression of key gene transcripts	Expression of genes highly regulated by Egr3 with restricted expression to developing spindles	Albert et al.[44]
Sensory neuron innervation	Sensory neuron ASW and FSE co-localisation with myotubes	Qiao et al.,[55] Guo et al.,[59] and Rumsey et al.[60]

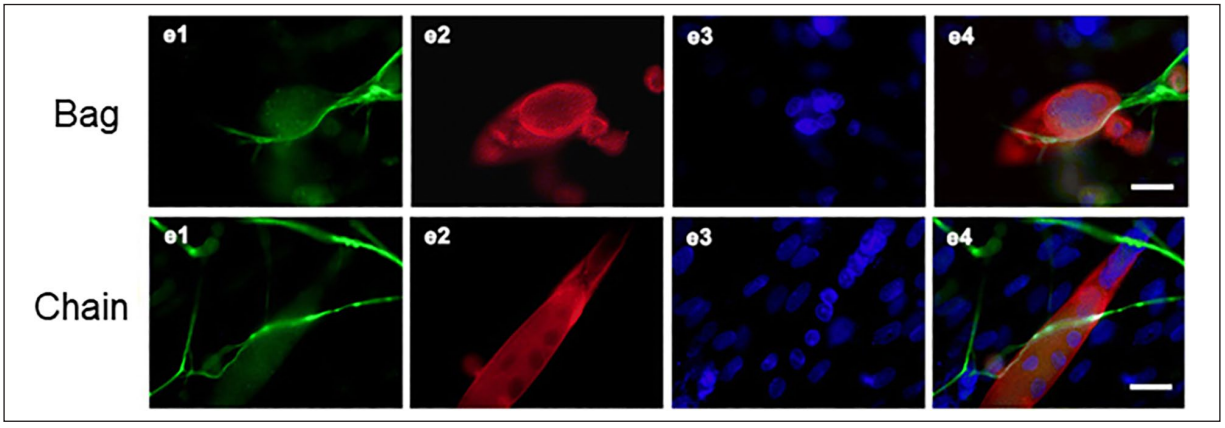

Figure 4. Bag and chain fibres visualised in a co-culture of DRG explants and dissociated skeletal muscle cells: e1 NF-200 immunoreactive neurons, e2 SKM cells, e3 DAPI, and e4 overlay of e1, e2, and e3. Scale bar = 20 μm. 'Bag', a–e Intrafusal nuclear bag fibre (red) with gathered nuclei (blue) inside and sensory nerve terminals wrapping around the bag fibre surface. 'Chain', a–e Intrafusal nuclear chain fibre (red) with linear assembled nuclei (blue) inside and sensory nerve terminals wrapping around the chain fibre surface.
Source: Figure taken and edited from Qiao,[55] Published through BioMed central and with permission of the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

expression is controlled at the transcriptional level, utilising mRNA transcriptional profiling will also greatly overcome issues relating to the use of antibodies. Table 2 displays the current extent of human myosin heavy chain genes (MYH) and the corresponding phenotype.[13,84,86]

Structure and role of the extracellular matrix

Muscle fibres reside within a three-dimensional scaffold consisting of numerous glycoproteins, collagens, proteoglycans and elastin, known as the extracellular matrix (ECM). The ECM contributes to the regulation many key physiological processes of skeletal muscle development, growth, repair and contractile function.[87–89] To date, *in vitro* models of intrafusal fibres are in a 2D environment, which alongside many limitations discussed in the future work section below, do not account for cell interactions with the ECM. The MS is enveloped by a capsular sleeve with two cellular portions, an outer capsule, that encloses a peri-axial space and an inner capsule that defines the

Table 2. Myosin heavy chain profiles: Nomenclature and phenotypes (modified from Schiaffino and Reggiani[84]).

Functional phenotype	Gene	Protein alias
Fast isoforms	MYH1	MyHC-IIx/d
	MYH2	MyHC-IIa
	MYH4	MyHC-IIb
Embryonic isoforms	MYH3	MyHC-embryonic
	MYH8	MyHC-neonatal
Slow/cardiac isoforms	MYH6	MyHC-α
	MYH7	MyHC-β
Specialised/ other isoforms	MYH13	MyHC-extraocular
	MYH7b/14	MyHC-slow/tonic
	MYH15	MyHC-15
	MYH16	MyHC-masticatory

axial compartments, where the intrafusal fibres and their nerve endings reside.[90,91] A basal lamina surrounds individual intrafusal fibres and the perineural epithelium of the outer capsule,[92] and unlike neuromuscular junctions, which lack basal lamina where the axon and muscle fibre meet, myo-sensory junctions have a common basal lamina that surrounds both intrafusal fibres and sensory terminals. The region has a modified structure and ECM composition in comparison to non-sensory regions.[93] There are reports of capsular thickening of the MS capsule in several neuromuscular disorders, aging[25,94] and as described previously, in adult mice ablated of satellite cells.[72] The specialised physiology and subsequent degradation in diseases associated with MS dysfunction, further suggests the MS ECM plays a significant role in muscle spindle function.[95]

Collagen IV has been proposed as a key ECM component to intrafusal development, as it is a significant component of the basal lamina surrounding muscle spindles.[56,92] However, it has been demonstrated that coating coverslips with Collagen IV, resulted in no measurable differences in NRG-1 induced intrafusal specific differentiation of primary rat myocytes *in vitro*.[57] This lack of effect, may be a result of the 2D environment, where cells on coated coverslips will respond and interact differently with themselves and their environment compared to cells in 3D, leading to distinct variation in mechanical and chemical cues, cell adhesions and migration.[80] Interestingly, MS lamina proteins Laminin and Agrin, can independently induce the expression of Egr3 *in vitro* through interaction with the a-dystroglycan receptor. The resulting expression is induced through the same ErbB receptors as Nrg-1, indicating an important role of the ECM in intrafusal fibre development, independent of Nrg-1.[96] The challenge ahead is to precisely identify the cell–ECM interactions involved and how the connective tissue ECM orients and regulates the intrafusal fibre phenotype. Candidates such as Collagen VI,[97] chondroitin sulphate proteoglycan, laminin, heparan sulphate proteoglycan and vimentin, show strong immunostaining in avian or mammalian MS extracellular spaces,[92,93,97,98] suggesting a strong role for these proteins in the development and maintenance of the MS. *In vitro* models investigating the functional role of MS ECM in its physiology, regeneration and degeneration are required to provide further understanding of how it could contribute to MS dysfunction in disease and aging. This can be achieved through the use of tissue engineered models, similar to that developed by our group and others (reviews[99–101]).

Functional responses

In addition to characterisation outlined in Table 1, functional responses of the MS are key in determining if an *in vivo*-like physiological response is achieved *in vitro*. To this end, a short summary of the electrophysiological responses will be highlighted.

Using electro-physiological techniques, the firing patterns as muscles stretch have been well-characterised experimentally in various mammals[102–109] and in humans *in vivo*.[110–113] Such analyses have also been replicated *ex vivo* and in explanted *in vitro* conditions.[74,114–118] To summarise, type Ia and II exhibit different dynamic responses to stretch, producing a unique patterns of discharge rate during stretch (Figure 5). Type Ia afferents display a burst of high impulse

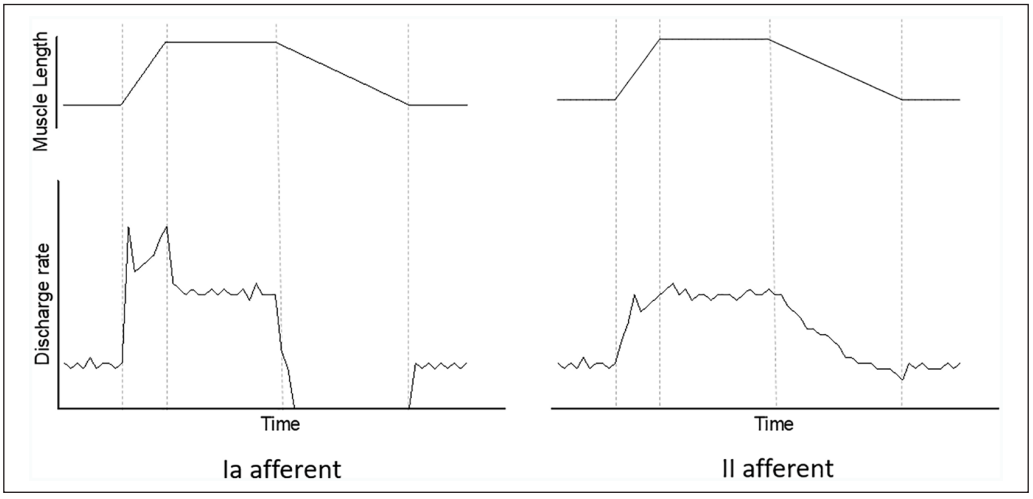

Figure 5. Formulated figure illustrating typical firing patterns for muscle spindle type Ia and II afferents, respectively. Discharge rate (imp/sec), muscle length (joint angle position or mechanical stretch) and time are on linear scales. Type Ia afferents display a burst of high impulse rate at the beginning of the ramp stretch, a transient fall in discharge rate at the beginning of the hold phase and an absence of impulses during most of the shortening. In comparison, type II afferents keep a steady firing pattern throughout the ramp hold stretch which correlates with the length of the muscle. The dotted lines from left to right show beginning of ramp stretch, hold phase, muscle shortening and finally the end of muscle shortening with a return to resting discharge rate.
Source: Figure adapted from Crowe and Matthews,[106] Kakuda and Nagaoka[110] and Edin and Vallbo.[122]

rate at the beginning of the ramp stretch, a transient fall in discharge rate at the beginning of the hold phase and an absence of impulses during the majority of shortening. In comparison, type II afferents keep a steady firing pattern throughout the ramp hold stretch which correlates with the length of the muscle. This firing pattern is not dependent on fusimotor innervation, as this pattern of response is elicited in de-efferented spindles.[119] There are two functionally distinct fusimotor fibres, static and dynamic, categorised by the excitatory effect upon afferent endings.[120] Stimulation of either fusimotor increases type Ia afferent firing rate at a constant length, with a greater increase in firing rate observed with static fusimotor stimulation. During the dynamic phase of stretching, the Ia afferent ending fires at a much higher rate when the dynamic fusimotor is stimulated compared to the static fusimotor, in which the dynamic response was lost, replicating a response similar to a type II afferent ending.[106,109,120,121]

Both sensory and motor neurons have been individually co-cultured with intrafusal fibres *in vitro*.[60,61] Afferent and fusimotor connections have been morphologically characterised through identification of ASWs and FSEs endings, localised on intrafusal fibres with co-localisation with brain sodium channels 1 and 2 (BNaC1 and BNaC2) and protein kinase C alpha (PICK1), proteins associated with the mechanosensory endings in non-mammalian studies.[60]Ca^2+ flux has been shown to be important for muscle spindle afferent activity *in vivo* and was therefore used to visualise a functional connection between intrafusal fibre and afferent neuron.[123] Afferent electrical activity was also visualised, following field stimulation to initiate myotube contraction, in afferent-intrafusal fibre co-cultures but absent in afferent only controls.[60]

In addition to mechanically stimulated action potential responses, the processes of mechanotransduction could be utilised to assess the intrafusal phenotype *in vitro*. Stretch-sensitive channels are responsible for transducing mechanical stimuli in spindle afferents. Most mammalian mechanosensory channels await definitive identification, however, ENaCs,[114] ASICs,[114,124] TRPs, Piezo 2[125] and Tentonin 3[126] have been associated with mechanosensory function, with strong evidence of sensory terminal localisation within the MS for ENaC and ASIC proteins.[124,127] One of the most compelling candidates is Piezo 2, a nonselective cation channel, which when ablated from sensory neurons in mice leads to severe proprioceptive defects and absent stretch evoked afferent responses to stretch in over 85% of muscles.[125] This suggests Piezo 2 is a principal mechanically activated ion channel required for physiological proprioception mediated through the muscle spindle.

Clusters of synaptic-like vesicles (SLVs) are a feature of muscle spindle primary afferent terminals when visualised via electron microscopy.[127] Furthermore, several functionally important pre-synaptic proteins are expressed at the spindle primary afferent terminals. Including vesicle clustering protein synapsin I, synaptophysin, vesicle docking SNARE complex protein, syntaxin 1B and many presynaptic Ca^2+ binding proteins; calbindin-D28k, calretinin, neurocalcin, NAP-22 and frequenin.[127,128] There is evidence of tonic Ca^2+ dependent, glutamate releasing, SLV exo/endocytosis, which significantly increases with mechanical activity[128] These data suggest a Ca^2+ dependent synaptic/secretory vesicle turnover system as part of MS mechanotransduction. Although a full review of this topic is beyond the scope of this article (see Bewick and Banks[127] and Bewick[128] for more details), it does highlight novel *in vitro* targets for displaying functionality in an innervated MS model. But also, how a sophisticated model will also offer a platform to help further define mammalian sensory mechanotransduction.

Fusimotors form cholinergic acetylcholine (ACh)-based neuromuscular interactions that appear in some aspects, similar to the neuromuscular junction formed by α-motoneurons on extrafusal muscle.[129,130] Therefore, functional fusimotor synapses with intrafusal fibres has been demonstrated using glutamate, an excitatory neurotransmitter capable of stimulating neurons in vitro.[131] Myotube action potentials (APs) were induced in the majority of the supposed innervated intrafusal fibres upon glutamate addition, which were not replicated in non-innervated intrafusal controls. The addition of curare, a competitive antagonist of ACh receptor, also caused immediate cessation APs.[61]

Future electrophysiological assessments should also focus on direct afferent firing in response to stretch and the effects of type specific fusimotor stimulation, trying to replicate the *in vivo* responses. This topic will be further discussed in the following section.

Future directions and challenges

Although there has been impressive recent progress in engineering and characterising intrafusal fibres *in vitro*, there are multiple avenues for researchers to consider (Figure 6), which have not been fully discussed in the sections above.

The greatest area for development, is the translation of current 2D progress towards more advanced 3D tissue engineered models. Cell behaviour is dependent on the niche they inhabit and external cues they receive, a 3D model better replicates the in vivo environment, offering a geometric similarity, allowing cells to interact with other cells and the surrounding matrix in three directions. The user can also control parameters such as stiffness, orientation, and porosity, all of which effect cell proliferation, migration, self-renewal and differentation.[132] 3D muscle cultures give rise to more mature molecular, structural and functional differentiation, characteristic of native adult muscle.[133–136] They also permit far longer

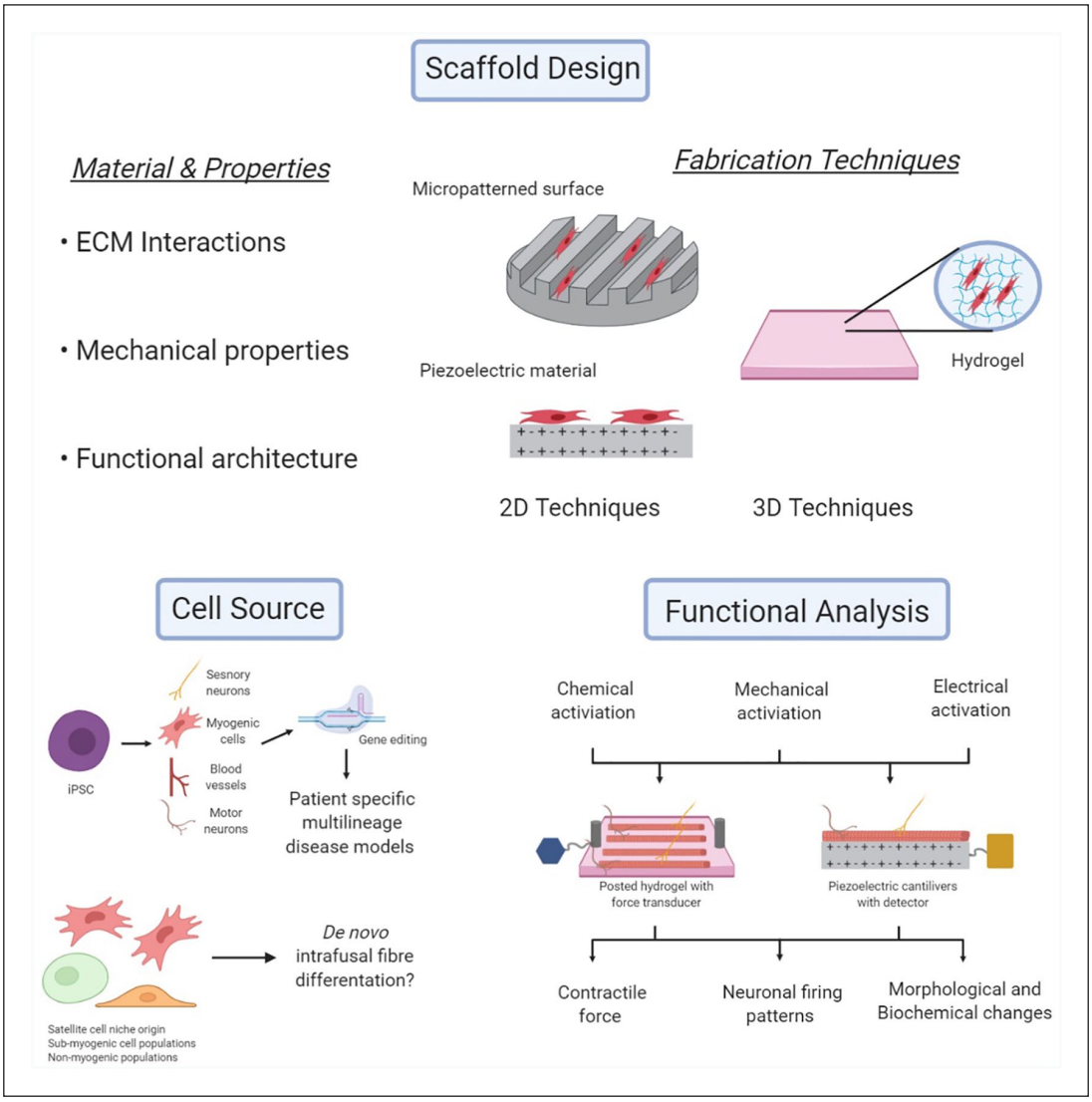

Figure 6. Schematic representation summarising key future research areas that can be explored to improve tissue engineering and characterisation of intrafusal skeletal muscle fibres.
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culture times,[134,137,138] support higher expression of adult MyHc isoforms and Ca^2+ handling genes, display increased alignment and hypertrophy, form developed sarcomeres and triads[101,134] and demonstrate basic contractile physiological properties such as positive force-frequency and length-tension.[89,101,134,139,140] From this, we can assume intrafusal fibres engineered in a 3D, aligned microenvironment, should result in a more mature phenotype and hence researchers should seek to develop and utilise these models.

Further structural advances include the morphogenic patterning of differentiating cells,[141,142] using physical and chemical patterning. Indeed, it is possible to control the freeform morphology of neurons using polymer brushes, which allows control at the micro-level (20μm).[143] Physical patterning methods (such as the use of electrospinning polymers, 3D printing, focussed ion beam and other micro-patterning methods) can also be used to generate surfaces on materials with micro- and nano-patterned features, which alters myogenic differentiation.[144–151] These methods could be used to chemically and/or physically pattern the necessary 'morphological template' for the differentiation of intrafusal fibre subtypes (i.e. bag and chain).

It is essential that future models of intrafusal fibres use functional assays to further validate and characterise the phenotype. It has been described extensively in this manuscript that the function of the muscle spindle is only possible where intrafusal fibres synapse with both sensory and motor neurons, creating an integrated feedback and feedforward system. Neuronal innervation of intrafusal fibres generated *in vitro* is therefore of paramount importance, when trying to recapitulate the *in vivo* structure.

The previously published methods to assess *in vitro* intrafusal fibre function, have assessed the electro-chemical and synaptic properties.[57,59,61] However, to facilitate physiological analysis of the model system, the platform would also need to support both mechanical stretch and load and permit for direct recording of afferent fibres and stimulation of fusimotor fibres. This could be achieved through the use of biomaterials (either natural or synthetic polymers) in a planar environment or the use of a 3D hydrogel.[101,133,135]

Biomedical microelectromechanical systems (BioMEMS) devices measure small scale mechanical movement, often relying on microcantilever sensors and can serve as a powerful functional assay for tissue engineered skeletal muscle.[152] A promising avenue for BioMEMS in muscle tissue engineering is piezoelectric materials, which can transduce mechanical stress to generate surface charges (or the inverse). Therefore, they can be used sense and quantify muscle contraction,[153,154] or as an actuator to apply mechanical force to cells[152,155] in real time with high throughput. Intrafusal models that facilitate direct afferent recording could be further utilised to characterise afferent Ia to type II depending on firing patterns observed during stretch. In addition, fusimotor stimulation permits the categorisation of static and dynamic fusimotors, depending on its excitatory effect upon afferent endings.[109,120–122] This integrated model could be used to investigate the relationship between afferent feedback, fusimotor feedforward and intrafusal fibre type in various diseased or damaged states.

The majority of publications in this area to-date, have utilised a tissue digestion methodology for the isolation primary muscle derived cells (MDCs).[55–57,59–61] As such, there is no control over the origin of the satellite cell progeny; these cells could be derived from the extrafusal tissue, or indeed from within the muscle spindle niche. It is currently unknown whether there are differences in the propensity for intrafusal differentiation of myoblasts derived from these two anatomically distinct regions. It also important to consider, that MDCs *in vivo* are a heterogeneous population of cells containing myogenic and non-myogenic (fibroblasts, pericytes, vascular endothelial cells, mesenchymal stromal cells, fibro-adipogenic progenitors, immune cells etc) populations.[156–158] Evidence suggests non-myogenic cells play an important supportive role in myogenesis,[157] therefore, future work should seek to identify the extent to which the non-myogenic populations contribute to intrafusal differentiation.[158–160] Additionally, there are skeletal muscle cell populations with myogenic potential, other than satellite cells[158,161] (mesoangioblasts, endothelial progenitor cells, adipose-derived stem cells, $CD133^+$ muscle derived stem cells etc.). Many are capable of *in vitro* myogenic differentiation,[161] but fail to rescue myogenesis following injury in the absence of satellite cells, suggesting a supporting role.[158] They have attracted interest as a candidate for myogenic cell transplantation therapies, proving a possible alternative to the progeny of satellite cells, which have historically been largely inefficient.[158,161,162] These sub-myogenic cell populations should be investigated for their ability for *de novo* intrafusal fibre differentiation.

Developments in hiPSCs and human amniotic mesenchymal cells (hAMCs) offer an exciting alternative to primary MDCs, they have the ability to self-renew and differentiate in a controlled manner into myogenic cells. iPSCs and hAMCs largely overcome issues relating to primary MDCs (large variability, limited passage capacity, ethical issues etc.) and offer an extremely powerful tool in personalised regenerative medicine, disease modelling and developmental biology.[163–165] Work with hAMCs in skeletal muscle tissue engineering is still limited, initial work has shown them as a suitable stem cell source for *in vitro* skeletal muscle tissue engineering and a viable method to treat volumetric tissue loss within the rat, causing increased angiogenesis and improved local tissue repair 4 weeks post implantation.[165] hiPSCs have been under the spotlight since there discovery in 2006,[166] they are generated by reprogramming donor somatic cells into a pluripotent state, which can then be reprogrammed to the desired cell type[164] and easily edited using genome editing tools such as CRISPR/Cas9 system.[167] hiPSCs represent an ideal source to produce patient and disease-specific adult cells for cell therapy and disease modelling. Indeed 3D hiPSC derived, multilineage muscle models containing myotubes, vascular endothelial cells, pericytes, and motor neurons, has been recently achieved.[168] Integration of hiPSC developed sensory neurons[169,170] and intrafusal fibres[58] into such a model, would provide the foundation of producing a complex human derived biomimetic tissue engineered model of skeletal muscle, that unlike any to date, will account for the afferent function of the muscle spindle.

Conclusion

There has been significant progress towards the generation of intrafusal fibres *in vitro*. This work has often been limited to the basic science of development and fundamental cell engineering. With the rapid and continued progress in tissue-engineered skeletal muscle models, there is an opportunity to develop anatomically and physiologically relevant models of the MS. These models could be used for developmental or disease state studies, pre-clinical screening of therapeutics, or clinical applications such as tissue replacement and regeneration for diseased and dysfunctional tissue.

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