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Skeletal muscle in a dish: towards making skeletal muscle in vitro

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chapter
01

Introduction

Introduction: Recreating Human Skeletal Muscle In Vitro

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Skeletal muscle is the most abundant tissue in the human body, comprising the largest tissue mass (40-50%). Skeletal muscle is vital for maintaining posture and enabling voluntary movement. It also protects soft tissues and regulates metabolic and homeostatic functions within the human body.[1,2] Anatomically, each muscle bundle consists of striated myofibrils, which are elongated, multinucleated cells encapsulated by a basal lamina. The basal lamina is divided into three different layers of extracellular matrix: the endomysium, perimysium, and epimysium (Figure 1).[3] Multiple myofibrils come together to create muscle fibers. The ECM layer that collects these muscle fibers is the endomysium. The unit formed by this encapsulation is called a fascicle or muscle bundle. The perimysium surrounds each fascicle or muscle bundle. The epimysium wraps the outer layer of skeletal muscle. Human skeletal muscle fibers exist in different isoforms. We observe that muscle fiber isoforms vary among different muscle groups. This reflects the specific roles of each isoform per different muscle group.[4] The development of muscle structures begins at the earliest stages of human embryogenesis. Skeletal muscle is derived from mesoderm, one of three embryonic germ layers.[5–7]

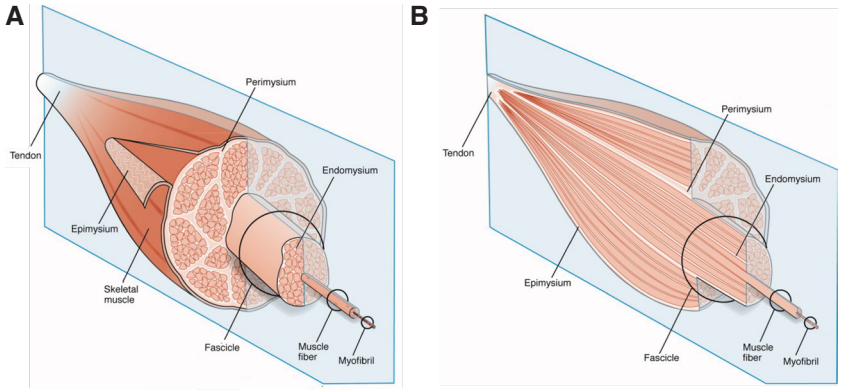

Figure 1–Schematic Overview Skeletal Muscle Anatomy. A –Skeletal muscle extracellular matrix divided into epimysium (surrounding the muscle), perimysium (surrounding muscle fascicles), and endomysium (surrounding individual muscle fibers). B – Cross-section of muscle tissue. *(Figure adapted from Gillies et al.[8])*

Early embryogenesis

In early human embryogenesis, a zygote is formed when a sperm cell fertilizes an egg cell. After a series of rapid cell divisions, the blastula is formed. The blastula is a one-dimensional layer of cells that transforms into a multilayered and multidimensional structure known as the gastrula. Next, in the process known as gastrulation, the three primary germ layers, endoderm, mesoderm, and ectoderm (Figure 2) form.[9]

The ectoderm is the outermost layer of the gastrula. Ectodermal cells give rise to the central and peripheral nervous systems. The ectodermal cells also form the outer layer of the human body, the epidermis. Additionally, ectoderm gives rise to neural crest cells. Neural crest cells migrate throughout the embryo to form sensory ganglia, melanocytes, and craniofacial cartilage.[10] The neural tube, also derived from the ectoderm, subsequently develops into the brain and spinal cord.[11] The most important pathways involved in ectodermal differentiation are the Bone Morphogenetic Protein (BMP) pathway and the Wnt pathway. Inhibition of BMP signaling is crucial for the formation of the neural plate, which is the precursor to the central nervous system. [12,13] The key genes that play a role in ectoderm development are SRY-box transcription factor 2 *(SOX2)*, Paired box protein Pax-6 *(PAX6)*, and Orthodenticle homeobox 2 *(OTX2)*. These genes are particularly important for neural specification during differentiation.[14–16]

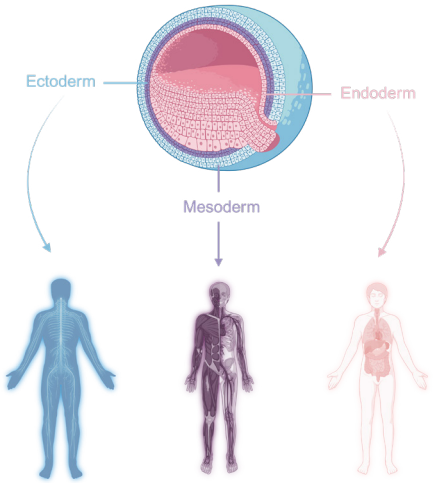

Figure 2 –Schematic Overview Embryonic Development. The outer layer of the gastrula contains the ectoderm and gives rise to nervous system. The middle-layer of the gastrula gives rise to mesoderm which gives rise to the circulatory system and the musculoskeletal system including the connective tissue. The inner-layer of the gastrula contains the endoderm which mainly forms the gastro-intestinal and urinary systems. *(Generated with BioRender.com)*

Underneath the layer of ectoderm, the mesoderm forms. The mesoderm is the germ layer that gives rise to the skeletal muscle, the skeleton, the cardiovascular system, and to urogenital structures. The most important pathways involved in mesodermal differentiation are the Transforming Growth Factor-beta (TGF-β) pathway, the Fibroblast Growth Factor (FGF) pathway, and the Notch pathway[17–19]. The pathways guide the mesoderm to specialize into three subdivisions: the paraxial mesoderm, responsible for the development of somites that later become vertebrae, skeletal muscle, and dermis; the intermediate mesoderm, which contributes to the formation of kidneys and gonads; and the lateral plate mesoderm, which forms the heart, blood vessels, and linings of body cavities (Figure 3).[20–22] Mesogenin1 *(Msgn1)*, Brachyury *(T)*, Mesoderm posterior 1 *(MESP1)*, and T-Box Transcription Factor 6 *(TBX6)* are the most essential genes in the development of mesoderm. The aforementioned genes also play an important role in mesodermal patterning and differentiation of further derivates.[23,24] Finally, the mesoderm also generates the notochord, a cylindrical structure that provides axial support and secretes signals to impact the differentiation of neighboring tissues.[9,25]

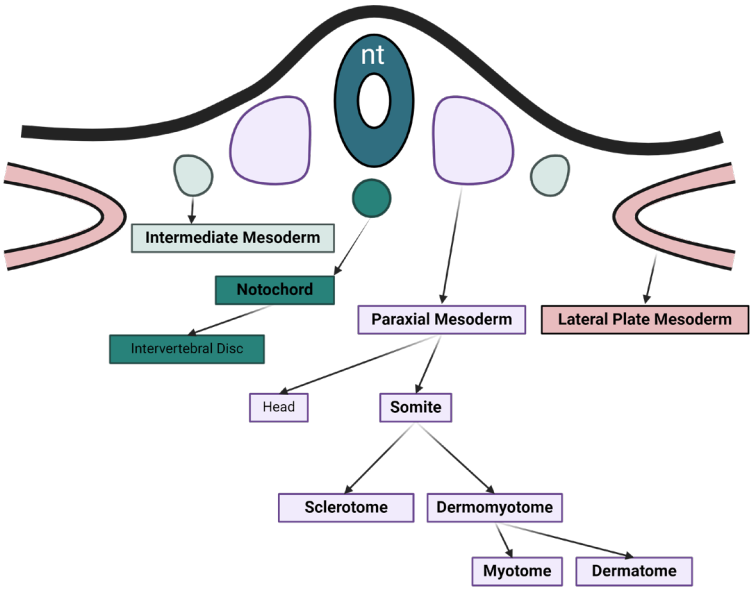

Figure 3 –Schematic Overview Mesoderm Derivates. Mesoderm divides into intermediate mesoderm, paraxial mesoderm and lateral plate mesoderm. Paraxial mesoderm differentiates into somites that divide into sclerotome and dermomyotome. The dermomyotome continues to divide into myotome and dermatome which eventually become skeletal muscle. The notochord provides signals or differentiation and continues to develop into the intervertebral disc. Nt: neural tube *(Figure adapted from Gilbert, S. F. & Barresi, M. J. F. Developmental Biology[9]).*

The innermost layer, the endoderm, is responsible for forming the epithelial lining of the digestive and respiratory systems and organs, including the liver, pancreas, and lungs. The pathways regulating endoderm differentiation are the Nodal signaling pathway, which is part of the TGF-β superfamily, and the Wnt and FGF pathways. The primary genes involved in endoderm formation are SRY-box 17 *(SOX17)*, Forkhead box protein A2*(FOXA2)*, and GATA Binding Protein 4 *(GATA4)*.[26] During embryogenesis, the endoderm folds to create the primitive gut, which differentiates into foregut (esophagus, stomach, and portions of the liver and pancreas), midgut (small intestine and parts of the colon), and hindgut (distal colon and rectum).[26]

A comprehensive understanding of the formation and differentiation of the ectoderm, mesoderm, and endoderm has been foundational to extensive research in developmental biology, providing critical insights across various disciplines.

Skeletal Muscle Myogenesis

Paraxial mesoderm formation

The paraxial mesoderm develops as an unsegmented strip of tissue next to the neural tube and notochord. Noggin, a BMP antagonist produced by the notochord, plays a crucial role in determining mesodermal cell fate by inhibiting differentiation into intermediate and lateral plate mesoderm. Concurrently, Wnt signaling, particularly involving Wnt3a and β-catenin, is essential for maintaining paraxial mesoderm progenitors. In the posterior region, this unsegmented tissue is known as the pre-

somitic mesoderm. Loss of function in crucial transcription factors downstream of Wnt signaling, such as *T*, *Tbx6*, and *Msgn1*, leads to defects in mesoderm formation, affecting segmentation and somitogenesis.[24,27]

As development proceeds, a spatiotemporal wave of segmentation occurs along the anterior-posterior axis, leading to the formation of somites in the anterior region, which defines the embryonic segments and is key to somitogenesis.[28] Segmentation in somitogenesis operates under a clock and wavefront model, where the segmentation clock involves oscillating gene expression cycles, notably governed by Notch, Wnt, and FGF pathways. These genes regulate the periodic formation of somites. The "wavefront" corresponds to a gradient of signaling molecules that move along the anterior-posterior axis, dictating somite formation. As cells pass through this wavefront, they receive signals to form distinct somites.[29] Key genes like *Hes7* regulate this process through negative feedback mechanisms.[30,31] *Mesp2*, activated by Notch and regulated by FGF, is crucial for somite segmentation and rostrocaudal patterning, establishing somitic boundaries, and upregulating Eph/ephrin signaling. This pathway mediates cell-cell communication for somite separation into individual segments facilitated by Eph receptors and their ephrin ligands.[32–34]

Following their formation, somites undergo compartmentalization along the dorsoventral axis, forming the dorsal epithelial dermomyotome and ventral mesenchymal sclerotome. The dorsal epithelial dermomyotome expresses Pax3, which differentiates into the myotome and the dermatome components[35]. The myotome contributes to musculature formation in the back, rib cage, ventral body wall, and limbs, while the dermatome forms the dermis of the back.[36,37] The ventral sclerotome contributes to the axial skeleton and associated tendons. Each somite initially comprises two compartments: an anterior compartment marked by T-Box Transcription Factor 18 *(Tbx18)* expression and a posterior compartment characterized by UNC homeobox *(Uncx)* expression, each leading to different developmental outcomes.[38] The initiation of myogenesis is signaled by the activation of myogenic factor 5 *(Myf5)* within the newly formed somite.[39,40]

Myogenesis

Myogenic cells begin to migrate out of the somite during limb buds formation, typically after specification of the dermomyotome and initial formation of the myotome. Skeletal myogenesis begins with myogenic cells originating from the dermomyotome lips, which differentiate to form primary muscle fibers. Subsequently, a progenitor population expressing *Pax3* and *Pax7* emerges from the central portion of the dermomyotome and persists throughout skeletal muscle development. Later in fetal stages, these progenitors populate satellite positions around myofibers, characterized by *Pax7* expression.[35,41,42]

Skeletal muscle myogenesis occurs in two phases: primary and secondary myogenesis (Figure 4 and Figure 5). Primary myogenesis takes place during the embryonic phase within the dermomyotome. The first postmitotic skeletal muscle cells to form are the myocytes, expressing specialized cytoskeletal proteins, such as slow *(type I, Myh7)* and embryonic *(Myh3)* myosin heavy chains *(MyHC)*, α-actins, and desmin.[43–45] These mononucleated myocytes align along the anterior-posterior axis of the somite.[46] New myocytes fuse with the aligned myocytes to form slow *MyHC*$^{+}$ myofibers.[47] Additionally, *Pax3*-expressing cells migrate to the myotome, contributing as myogenic precursors for a later stage in myogenesis[48].

The secondary myogenesis occurs during the fetal stage and allows newly formed myotubes to mature into myofibers. The myogenic program in the trunk and limbs is tightly controlled by core transcription factors, including *Pax3* and muscle regulatory factors (MRFs), such as *Myf5*, *MyoD (Myod1), MRF4 (Myf6),* and *myogenin(Myog)*.[50–52] *Myog* specifically regulates the terminal differentiation of myoblasts into myocytes[53]. During the secondary phase of myogenesis, a subset of Pax3^{+} myogenic progenitors initiate expression of *Pax7* while downregulating *Pax3*. These *Pax7*$^{+}$ myogenic precursors subsequently fuse with each other or with primary fibers, giving rise to fetal fibers.

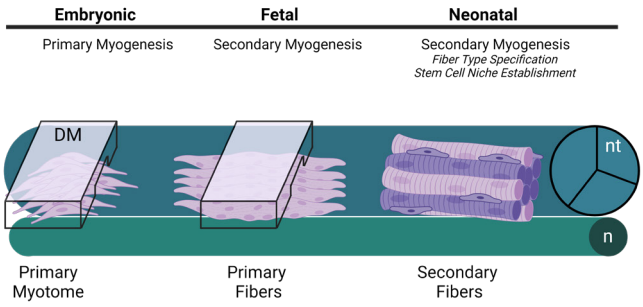

Figure 4 –Developmental Stages of Skeletal Myogenesis from Dermomyotome. Primary Myogenesis (left): Composed of primary myocytes aligned along the anteroposterior axis within each somitic compartment. Secondary myogenesis (middle and right): primary fibers form after fusion of Pax3+ progenitors. Some Pax3+ progenitors migrate from the ventral lip to populate the body wall and limb buds (not shown). Pax7+ myogenic form secondary fibers using primary fibers as a scaffold, contributing to fetal muscle growth. Satellite cell precursors localize under the basal lamina of fibers Abbreviations: nt: neural tube), n: notochord, DM: dermomyotome. *(Genereted with Biorender adapted from Chal et al.*[49]*)*

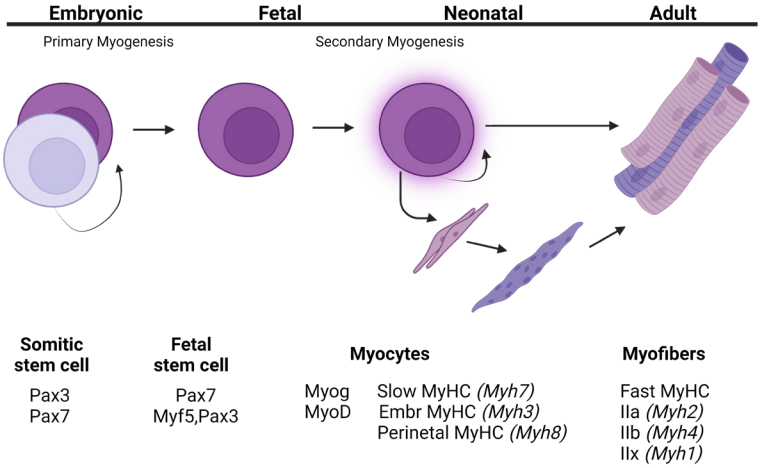

Figure 5 –Gene Markers of Stages of Skeletal Myogenesis . Myogenic stem cells contribute to fetal myogenesis while maintaining a progenitor pool, which eventually localizes on mature myofibers in the satellite cell niche. Markers for intermediates and differentiated skeletal myofibers are shown at each step. *(Generated with Biorender adapted from Chal et al.*[49]*)*

As this occurs, these myogenic cells start expressing fast *MyHC* isoforms and continue maturing into myofibers.[54] Additionally, a subset of these *Pax7*$^{+}$ progenitors

differentiate into adult muscle stem cells known as satellite cells, crucial for adult muscle regeneration and repair.[55–57]

During fetal and neonatal development, muscle fibers transiently express perinatal myosin heavy chain (MyHC, *Myh8*). As development progresses, fast isoforms of MyHC, such as types IIa *(Myh2)*, IIb *(Myh4)*, and IIx *(Myh1)*, emerge during late fetal myogenesis. In adults, muscle fibers express specific MyHC isoforms that correspond to their electrophysiological properties and metabolism. Oxidative slow-twitch fibers express slow MyHC (type I, *Myh7*), while glycolytic fast-twitch fibers express fast MyHC isoforms mentioned above. The expression of these MyHC isoforms follows a developmental sequence, starting with embryonic and slow MyHC, and is regulated by transcription factors like Six1 and Eya1, as well as neural stimulation during fetal development.[4,58,59]

The intricate process of myogenesis is essential for developing and maintaining skeletal muscle. This highly regulated process, as described, involves a network of transcription factors and signaling pathways, ensuring proper muscle growth and function. Genetic anomalies disrupting these regulatory mechanisms can result in a spectrum of skeletal muscle disorders, characterized by muscle weakness, degeneration, and impaired function. Understanding these genetic skeletal muscle disorders is crucial for developing effective therapeutic strategies.

Genetic skeletal muscle disorder

Skeletal muscle disorders, often referred to as myopathies, encompass a diverse group of conditions caused by genetic mutations that affect muscle structure and function. Among the most studied are Duchenne Muscular Dystrophy (DMD), Facioscapulohumeral Muscular Dystrophy (FSHD), Myotonic Dystrophy (DM), and Limb-Girdle Muscular Dystrophy (LGMD).

DMD is an X chromosome-linked disorder that affects approximately 1 in 5,000 males each year. The disease is characterized by progressive muscle degeneration and weakness, typically beginning in early childhood. Due to severe muscle deterioration, individuals with DMD often require assisted ventilation by around 20 years of age, reflecting the disease's impact on respiratory muscle function.[60,61] DMD is caused by mutations in the *DMD* gene, located at Xp21.2–p21.1. This gene encodes for dystrophin, a protein essential for the muscle sarcolemma and a key component of the dystrophin-glycoprotein complex, which is vital for the stability of the plasma membrane of striated muscle fibers. Defects in dystrophin compromise membrane integrity, leading to progressive degeneration and eventual loss of both skeletal and cardiac muscle tissues. These disruptions underly the muscle weakness and severe physical complications associated with DMD.[62,63]

FSHD is an autosomal dominant disorder linked to the contraction of the D4Z4 repeat on chromosome 4, which leads to the aberrant expression of the *DUX4* gene. This disorder primarily affects facial, shoulder, and upper arm muscles, with symptoms typically appearing in adolescence or early adulthood. Despite its slow progression, FSHD can cause significant functional impairment and reduced quality of life.[64]

DM is characterized by progressive muscle wasting, weakness, myotonia, and multi-systemic involvement, including cardiac, endocrine, and ocular systems. There are two main types of DM: DM1, caused by mutations in the *DMPK* gene; and DM2, resulting from mutations in the *CNBP* gene. Both types are autosomal

dominant and involve nucleotide repeat expansion, leading to toxic RNA products that disrupt normal cellular function.[65]

LGMD encompasses a variety of genetic disorders that primarily affect the muscles of the pelvic and shoulder girdles. It is part of the broader category of laminopathies caused by mutations in the LMNA gene encoding lamin A and lamin C proteins. LGMD can be inherited in autosomal dominant or recessive patterns, with symptoms ranging from mild to severe muscle weakness and atrophy. The specific genetic mutations in LGMD influence disease onset and progression. [66]

Advanced in vitro models are crucial to fully understanding the underlying mechanisms of genetic skeletal muscle diseases. Given the heterogeneous nature of many skeletal muscle diseases, a personalized approach is necessary, and in vitro models provide an ideal platform for screening potential therapies.

Skeletal Muscle in Three-Dimensional Models

The development and application of three-dimensional (3D) in vitro models for skeletal muscle tissue have revolutionized the field of tissue engineering. These models provide a more physiologically relevant environment compared to traditional two-dimensional (2D) cultures, enabling more accurate studies of muscle biology, disease mechanisms, and potential therapeutic interventions.[67] One primary advantage of 3D models is their ability to faithfully recapitulate the structural and functional characteristics of native muscle tissue. This includes mimicking the organization of muscle fibers, the composition of the extracellular matrix, and the mechanical properties that are crucial for studying muscle physiology and pathology in conditions closely resembling those in vivo.[68,69]

Additionally, the development of 3D skeletal muscle models addresses ethical concerns and regulatory pressures to minimize animal testing. These advanced in vitro models not only reduce reliance on animal models but also serve as high-throughput platforms for drug screening and toxicity testing, providing more reproducible and human-relevant data. By aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal research, these models promote more ethical and sustainable scientific practices[70,71]. They also help circumvent limitations of animal models, such as species-specific differences in disease manifestation and drug response. Additionally, using human cells in these models provides direct access to the human genome.

Investigating skeletal muscle in both healthy and diseased states using 3D models is essential for understanding the complex interactions between muscle cells and their microenvironment. These models have proven invaluable in studying various conditions, including muscular dystrophies, inflammatory myopathies, and other muscle-related disorders. [69,72,73] As mentioned above, significant differences between human and animal models, including variations in muscle physiology and disease mechanisms, can produce misleading results. For example, the efficacy of treatments such as the human monoclonal antibody tralokinumab in mice did not translate to success in human Phase III trials for asthma treatment.[74] Advanced 3D models offer a more accurate and ethical alternative, emphasizing the need for these models in developing and testing effective therapies for muscle diseases.

Furthermore, 3D models allow for the incorporation of mechanical and biochemical cues that are critical for cell differentiation and maturation. For example, studies

have demonstrated that applying mechanical stretch or electrical stimulation in 3D cultures promotes the maturation and alignment of muscle fibers, closely mimicking physiological conditions in vivo.[75–78]These cues are challenging to replicate in 2D cultures, underscoring the superiority of 3D models in simulating native tissue environments.

Another advantage of 3D models is their ability to support the co-culture of multiple cell types, which is important for studying tissue-tissue interactions and complex disease mechanisms. In skeletal muscle research, co-culturing muscle cells with endothelial cells, fibroblasts, or neural cells in a 3D environment provides insights into the interactions that regulate muscle function and pathology.[79–83] This complexity is challenging to achieve in 2D cultures, where the spatial organization and interaction dynamics are limited.

The application of 3D in vitro models extend beyond basic research to regenerative medicine and personalized therapies. For example, creating patient-specific muscle models using stem cells enables the study of individual genetic variations and personalized treatment responses[78,83–85]. These advancements could lead to more effective and tailored therapies for muscle-related diseases, offering a platform to test drug efficacy and safety on a patient-by-patient basis, potentially improving clinical outcomes and reducing adverse effects.

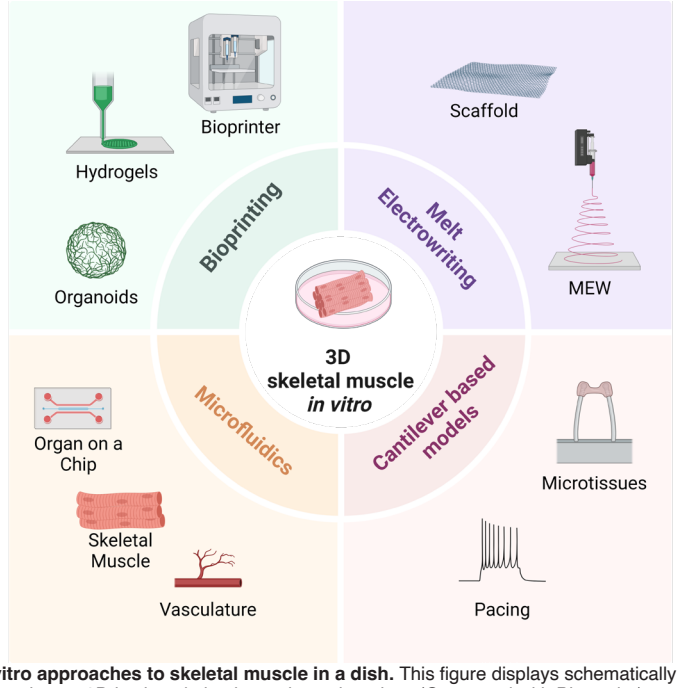

Figure 6 –3D in vitro approaches to skeletal muscle in a dish. This figure displays schematically the possibilities of four different approaches to 3D in vitro skeletal muscle engineering. *(Generated with Biorender).*

Approaches to 3D in vitro model: 3D printing

Creating 3D models can be achieved through the application of a range of emerging technologies. One notable example is 3D bioprinting, which has significantly advanced the development of in vitro skeletal muscle models. Bioprinting enables the precise fabrication of muscle tissues with controlled architecture and cellular composition, enhancing the physiological relevance of the models. This technique

incorporates various cell types, growth factors, and extracellular matrix components to create a more realistic muscle tissue environment. Bioprinted muscle tissues have been used to study muscle regeneration, drug responses, and the effects of mechanical stimuli on muscle function, providing valuable insights into muscle biology and potential therapeutic strategies.[86–89] Additionally, bioprinting can introduce vasculature into 3D models.[90]

Another printing technique that can be employed for 3D modeling is Melt Electro Writing (MEW). MEW is a cutting-edge additive manufacturing technique that fabricates highly defined microfibrous scaffolds. This technology uses a heated nozzle to melt a polymer, which is then electrostatically drawn into fine fibers. The fibers are deposited onto a collector in any predetermined shape. The polymer melt requires sufficient cooling to solidify either just before or immediately after the molten jet touches the collector, depending on the processing parameters. Unlike other electrostatic fabrication methods, MEW does not require solvents, which are often volatile and need to be removed post-printing, to make the polymer printable; this simplifies the process and enhances safety.[91]

The current gold standard polymer used for MEW is poly(ε-caprolactone) (PCL), a semicrystalline, biodegradable polyester approved by the U.S. Food and Drug Administration (FDA) for certain clinical applications. PCL's low melting temperature and rapid solidification properties make it highly suitable for MEW, offering a slow degradation rate that lasts for several years.[91,92] PCL is extensively studied in biomedical engineering and is used in various other manufacturing technologies due to its availability in different molecular weights, biocompatibility, and biodegradability[93]

MEW is predominantly used for scaffold production, serving as a base for the bio-assembly of 3D in vitro models. The precision of MEW allows for high-level regulation over scaffold fabrication with controlled fiber diameters and complex geometries that aid in guiding cell growth, cell alignment, and differentiation. Studies utilizing PCL have explored various designs, ranging from typical box-structured scaffolds and scaffolds for cell experiments to more complex structures like sinusoid designs with horizontal layer stacking, tubes, and fiber-hydrogel composites with mechanical properties similar to those of a heart valve.[94–97]

Despite the significant potential of PCL-based scaffolds, their ability to mechanically condition skeletal muscle cells remains limited. Exploring alternative materials that can be stimulated mechanically through methods such as magnetic or other types of external stimuli, may enhance the functional performance of MEW-based scaffolds for engineered skeletal muscle tissues.

Approaches to 3D models: cantilever-based models

A cantilever-based model is a system where cells embedded in a hydrogel are positioned between two attachment points, commonly referred to as pillars. The set up allows cells to self-assemble into tissues, while providing mechanical support. It also enables measurement of contractile forces as the cells exert tension on the hydrogel, causing the cantilevers to bend, which can then be quantified into generated forces.

Several research groups have successfully developed and used cantilever-based systems for skeletal muscle models by embedding differentiating human myoblasts into various hydrogels, including fibrin, collagen, and Matrigel. These experimental

setups aim to replicate the mechanical cues present in the native skeletal muscle niche, which are often absent in traditional 2D or even 3D organoid cultures. By providing embedded cells with a matrix for attachment and establishing a tension axis through the hydrogel between attachment points, these models facilitate the alignment of myotubes. This induced tension and alignment promote sarcomere maturation and reveal disease-specific phenotypes less apparent in 2D cultures.[76,77,98,99]

One of the pioneering cantilever-based models was developed in Thomas Eschenhagen's lab in 2010, initially aimed at creating engineered heart tissues (EHTs) for drug testing.[100] This model was later adapted from cardiac to skeletal muscle applications, enabling research into fundamental aspects like satellite cell roles within their niche and disease modeling.[83,101] This foundational design has served as a blueprint for numerous platforms. For example, Curi Bio's "Mantarray" system has integrated magnetic sensing of contractility into their cantilever-based system for engineered skeletal muscle tissues[102]. Another innovative example is Optics11's latest product "Cuore," which uses optical fibers to measure contractility, showcasing advancements in cantilever-based skeletal muscle modeling technologies.[103]

One disadvantage of the initial EHT design is its size, which limits scalability. Creating single tissues in these platforms requires a large number of cells, ranging from 500,000 to 1,500,000. Consequently, there is a significant trend within the tissue engineering field to miniaturize these systems into a 96-well format. This miniaturization reduces the required cell count per tissue to no more than 100,000, facilitating higher throughput and making the process more efficient for large-scale applications. While efforts within the skeletal muscle engineering field have successfully miniaturized engineered tissues [84,104–106], understanding how scaling down affects tissue development, cell behavior, and overall functionality remains a critical area for further research.

Cantilever models for engineering skeletal muscle are gaining prominence in the field and increasingly serve as the benchmark for 3D in vitro skeletal muscle disease modeling. They have been instrumental in studying diseases such as DMD [84,107], Pompe Disease[98,] and Laminopathies[108], as well as in research on aging[109] and regeneration[109,110]. Beyond disease modeling, cantilever models are vital for preclinical drug testing, providing a versatile and reliable platform for advancing our understanding of muscle physiology and the development of new treatments.[111]

In summary, 3D in vitro models offer numerous advantages over 2D models, including enhanced cell maturation, more accurate tissue architecture, and the incorporation of mechanical and biochemical cues. These benefits make 3D models a powerful tool for studying skeletal muscle biology, disease mechanisms, and therapeutic development. As tissue engineering continues to advance, 3D models are expected to play an increasingly important role in translational research and personalized medicine.

Cell sources for in vitro models

Both 2D and 3D in vitro models for skeletal muscle research predominantly utilize three main cell types: primary myoblasts, immortalized myogenic cell lines, and pluripotent stem cells. Compared to traditional 2D cell cultures, 3D in vitro models offer several advantages, particularly in enhancing the maturation of induced pluripotent stem cells (iPSCs)-derived myogenic cells. One significant benefit is the ability to provide a more physiologically-relevant environment that closely mimics

in vivo conditions. In 2D cultures, cells grow in a flat monolayer, which fails to represent the complex 3D architecture of tissues accurately. This lack of spatial organization can lead to differences in cell behavior, gene expression, and cellular interactions compared to cells in a 3D environment.[112]

In 3D models, iPSCs can differentiate into more mature and functionally relevant cell types. For example, 3D cultures have been shown to enhance the maturation of iPSCs into skeletal muscle cells, resulting in improved muscle fiber formation and functionality. [72] The 3D environment supports better cell-cell and cell-matrix interactions, which are crucial for the development of tissue-specific architecture and function.[112–115] Improved maturation of iPSC-derived myogenic cells is crucial for creating accurate disease models and developing effective therapeutic interventions. However, current protocols often yield cells with an immature phenotype, highlighting the need for further advancements to achieve fully mature and functional myogenic cells for these applications. [116]

Pluripotent Stem Cell-Derived Skeletal Muscle

Since their discovery by Yamanaka et al. in 2006, iPSCs have revolutionized regenerative medicine and disease modeling. iPSCs are generated by reprogramming adult somatic cells through the introduction of specific transcription factors: Oct4, Sox2, Klf4, and c-Myc, collectively known as the OSKM factors. [117] The generation of iPSCs can be achieved by activating the OSKM factors in somatic cells through viral vector delivery or non-integrated DNA or RNA molecules, which reprogram the cells to a pluripotent state.[118–120] This groundbreaking technique was first demonstrated in mouse embryonic fibroblasts and later applied to human cells.[117]

iPSCs have the capability to differentiate into any cell type from the three germ layers[121], making them invaluable for studying a wide range of diseases, developing personalized medicine, and advancing drug screening and toxicity testing. For example, iPSCs have been instrumental in modeling neurodegenerative diseases like Parkinson's disease, cardiovascular diseases, and liver disorders, providing insights into disease mechanisms and potential treatments.[122,123]

In skeletal muscle research, several labs have successfully developed protocols to differentiate human iPSCs into skeletal muscle progenitors[124–128]. These protocols enable the modeling of muscular dystrophies, the study of muscle development, and the development of cell-based therapies for muscle regeneration.[125,129,130] These iPSCs, capable of differentiating into various cell types, including various types of skeletal muscle progenitors, offer a versatile platform for exploring muscle development and regeneration in vitro. Understanding the in vivo formation of skeletal muscle provides crucial insights into potential methods for generating skeletal muscle in vitro, further enhancing our ability to study and treat muscle-related diseases.

In vitro, generation of skeletal muscle from iPSCs can be achieved through two methods: directed differentiation and direct reprogramming. Direct reprogramming, also known as transgenic reprogramming, involves overexpressing specific transcription factors that induce cells to adopt a myogenic lineage. Conversely, directed differentiation techniques aim to recapitulate early differentiation stages observed during embryonic development to generate skeletal muscle in vitro, primarily using biochemical cues through small molecules (Figure 7).

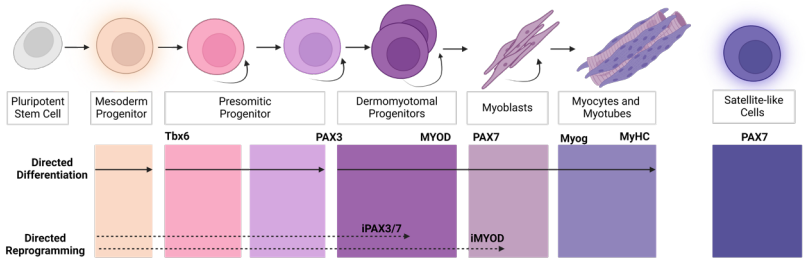

Figure 7 –Skeletal Myogenesis from Pluripotent Stem Cells. Comparison of two main strategies: directed differentiation and directed reprogramming. During directed differentiation, the pluripotent cell goes through the developmental stages of paraxial mesoderm specification and. With direct reprogramming approaches, the aim is to bypass early developmental stages by overexpressing a myogenic regulator, such as Pax3/7 or MyoD (iPAX3/7, iMYOD) *(Generated with Biorender, adapted from Chal et al.*[49]*)*

Directed reprogramming in myogenic differentiation often focuses on transcription factors, such as MYOD1, PAX3, and PAX7. The use of muscle-related transcription factor overexpression began with the discovery in the late 1970s that fibroblasts could be converted into muscle cells using 5-azacytidine, a non-specific demethylating agent. This agent was believed to target the MYOD1 locus, although its exact mechanism was not fully understood at the time.[131] In the late 1980s, Weintraub et al. identified *MyoD* as a master regulator of myogenesis. Overexpression of *MyoD*

in non-muscle cells such as fibroblasts could induce these cells to adopt a muscle cell fate, showcasing the *MyoD's* potential to reprogram the transcriptional landscape of cells and promote muscle-specific gene expression.[132,133]

MYOD1 overexpression at the onset of differentiation bypasses early embryonic stages, initiating myogenic induction from myoblast-like progenitors.[134–137] This approach, however, limits the study of early myogenesis and raises questions about the fidelity of reprogrammed cells in representing mature skeletal muscle, as they skip some myogenic developmental stages. Furthermore, the expression levels and duration of key factors normally present during myogenic differentiation may disrupt the system, potentially hindering the long-term maturation and functionality of these cells. Nonetheless, achieving efficient induction of MYOD1 expression, up to 90%, is remarkable.[138]

Another myogenic induction strategy involves overexpressing transcription factors, such as PAX3 and PAX7, which precede MYOD1 expression during embryonic development [139–141]. This approach is particularly valuable for generating a population of skeletal muscle stem cells suitable for transplantation[141,142].

Directed differentiation of iPSCs offers a non-transgenic approach, making it more suitable for clinical applications. By using small molecules that induce various signaling pathways, differentiation protocols aim to recapitulate embryonic development in vitro. Typically, pluripotent stem cells progress through mesodermal progenitor stages, with activation of Tbx6 directing progenitors to become presomitic mesoderm cells, followed by *Pax3* activation, leading to dermomyotome progenitor status and *MyoD* expression initiation. This sequence triggers myogenesis, guided further by Pax7 expression, facilitating the differentiation of dermomyotomal progenitors into myoblasts. Subsequent myogenin expression drives the maturation of myoblasts into myocytes, which fuse to form myotubes and myofibers, ultimately

expressing MyHC. A subpopulation of these cells remains Pax7-positive, serving as satellite cells, critical for muscle repair and regeneration.[49]

This directed differentiation method not only preserves early differentiation stages but also enables the in vitro modeling of early skeletal muscle development, providing valuable insights into muscle biology and potential therapeutic applications. However, its main drawback lies in the labor-intensive protocols spanning 25-50 days, resulting in heterogeneous cell populations. Yield and purity of iPSC-derived mesoderm can vary significantly, affecting the purity of muscle progenitors and thus limiting the efficiency and effectiveness of these cells for research.[143,144].

One approach to mitigate heterogeneity involves sorting the cells by fluorescence-activated cell sorting (FACS) based on an array of identified surface markers, such as VCAM1 (CD106, SM/C2.6), CD34, NCAM1 (CD56), CXCR4 (CD184), ERBB3, CD56, and CD82[124,128,145,146], although these markers are not exclusive to myogenic cells. This sorting strategy helps reduce heterogeneity in differentiation but requires further identification of key regulators of in vitro myogenesis to increase the final yield of directed differentiation.

The Role of Non-coding RNAs in Germ Layer Differentiation and Skeletal Muscle Differentiation

Initially, developmental biology focused predominantly on the coding regions of the genome, which constitute less than 3% of the total genome (Figure 8). The remainder was often dismissed as non-functional or “junk” DNA. However, advancements in sequencing technologies and omics approaches have revealed that non-coding regions of the genome play critical roles in regulating gene expression. [147] Recently identified families in these non-coding regions are long noncoding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs) that do not encode proteins, but have emerged as important regulators of various biological processes, including stem cell differentiation.

lncRNAs are transcripts longer than 200 nucleotides, typically transcribed by RNA polymerase II, often spliced, and polyadenylated [148]. sncRNAs, ranging from 20-30 nucleotides in length, include microRNAs (miRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), and piwi-interacting RNAs (piRNAs). These sncRNAs regulate gene expression through mechanisms including mRNA degradation, translation inhibition, and chromatin remodeling.[149] For example, snoRNAs are chemically modification other RNAs, particularly ribosomal RNAs (rRNAs), while tRNAs play a crucial role in translating mRNA sequences into proteins. piRNAs are key to silencing transposons and regulating gene expression in germ cells [150–152] , and LncRNAs can regulate miRNAs, and vice versa.[153]

To deepen our understanding of differentiation processes, advanced omics approaches, including advanced sequencing techniques that capture non-coding RNAs, are invaluable. While protein-coding genes have long been manipulated to induce differentiation of iPSCs, the discovery of thousands of ncRNAs highlights their potential in differentiation regulation. Therefore, there is growing interest in exploring these non-coding RNAs further, as they may hold critical insights into enhancing and regulating differentiation processes.

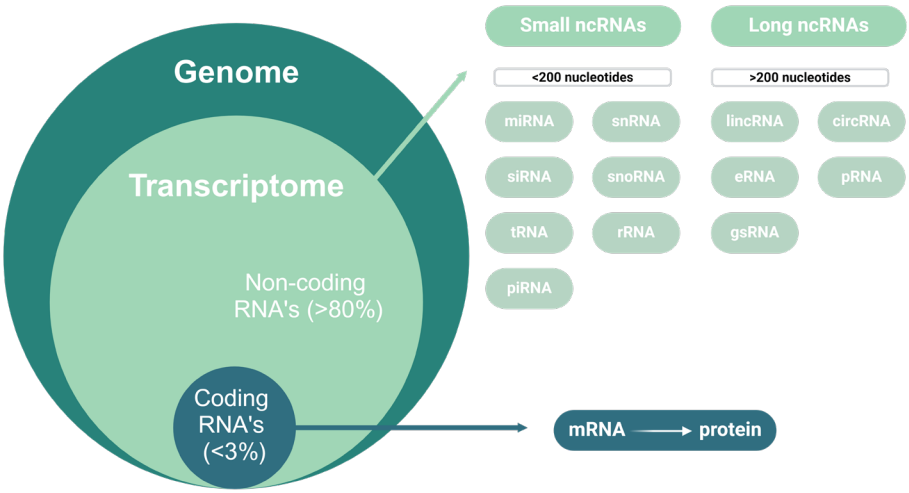

Figure 8 –Schematic overview of coding and non-coding RNA's in the human genome. The majority of the genome codes for non-coding RNA'S. *(Generated with Biorender)*

The role of non-coding RNAs towards myogenic differentiation

LncRNAs regulate the development of early germ layer tissues and have significantly benefited from large-scale sequencing efforts to identify functionally important transcripts. Figure 9 provides an overview of some known lncRNAs associated with each germ layer. We are in particularly interested in understanding the role of ncRNAs in mesoderm differentiation. As previously mentioned, a key regulator in mesoderm differentiation is *T-box transcription factor T*, also known as *Brachyury*. The neighboring gene, yin yang lncRNA T *(yylncT)*, represents a class of lncRNAs that are transcribed in a divergent manner that safeguards the T-mediated mesodermal commitment in human iPSCs. yylncT functions by binding to the *de novo* DNA methyltransferase DNMT3B, which prevents local DNA methylation at the yylncT/T locus, thereby enhancing T expression. Loss of *yylncT* significantly inhibits *T* and early mesodermal gene expression during mesoderm induction, while differentiation towards ectoderm or endoderm remains unaffected.[154]

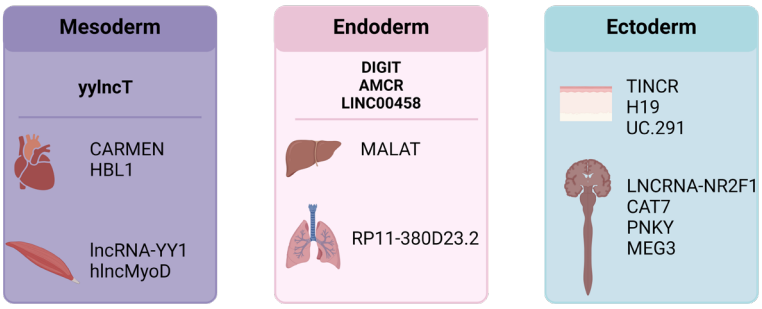

Figure 9– Overview of the lncRNAs with known roles in human germ layer differentiation. Mesoderm and its derivatives: heart and muscle(purple). Endoderm and its derivatives: liver and long (pink). Neuroectoderm and its derivatives: epidermis and nervous system (blue). *(Generated with Biorender and inspired by Azad at al.[157])*

In the context of myogenic differentiation, *linc-YY1* has been identified to promote this process through interaction with the transcription factor Ying Yang 1 (YY1)[155]. Further along the differentiation pathway, *lncMyoD* was shown to regulate MyoD expression by directly binding to IGF2-mRNA-binding protein 2 (IMP2). This interaction negatively regulates IMP2-mediated translation of proliferation genes, such as N-Ras and c-Myc, and allows for MyoD-mediated cell-cycle exit and subsequent myogenesis of myoblasts.[156]

The role of miRNAs in muscle development

MicroRNAs involved in muscle development or regulation are collectively called myomiRs. They are approximately 21 nucleotides long and function by binding to the 3'-untranslated regions (3'-UTR) of target messenger RNAs (mRNAs). This binding typically leads to mRNA degradation or inhibition of translation, thereby regulating gene expression. The most common myomiRs belong to the miR-1 and miR-133 families.

These miRNAs, including miR-1, miR-1-2, miR-206, miR-133a, and miR-133b, suppress the expression of non-muscle genes during stem cell differentiation. [158,159] In zebrafish, downregulation of miR-1 and miR-133 affects muscle gene expression and sarcomere assembly[160]. MyomiRs like miR-206 are detected in chick and mouse embryos and influence myogenesis via MRFs[161]. Other miRNAs, such as miR-196 and miR-203, also play significant roles in muscle development, illustrating myomiRs' critical regulatory function in muscle gene expression during embryonic development.[162]

Furthermore, it has been shown that the expression of lncRNA *Yam-1* increases the levels of microRNA-715, which regulates expression of Wnt7b, a protein that promotes muscle differentiation. Consequently, microRNA-715 acts as an anti-myogenesis factor[163]. Many more lncRNAs have been identified regulate myogenesis either by influencing the expression of protein-coding genes or other ncRNAs. An overview of the most important lncRNAs and miRNAs involved in myogenesis has been provided in Figure 10 adapted from Luo et al.[164]

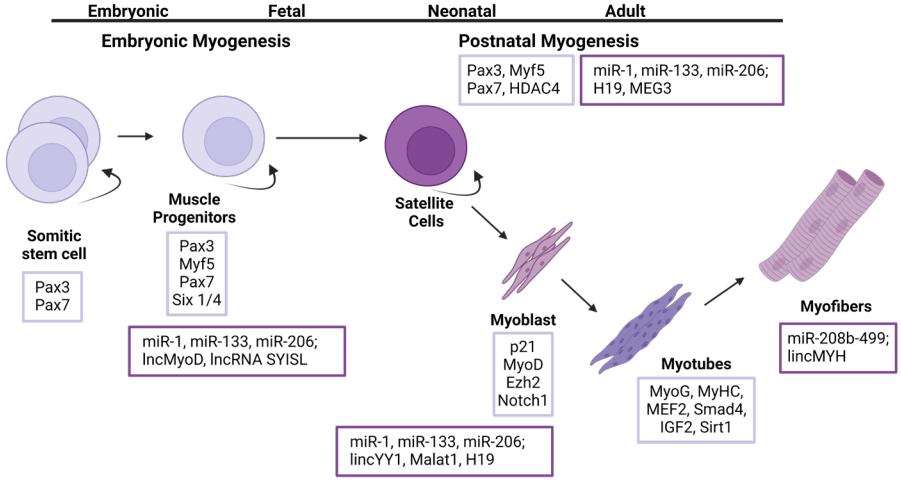

Figure 10 –Schematic overview of coding genes and ncRNAs involved in embryonic and postnatal myogenesis. Coding genes and ncRNAs regulate the process of embryonic and postnatal myogenesis. Coding genes are represented in light purple, and ncRNAs are represented in darker purple squares. (*Generated with Biorender and inspired by Luo et al.*[164])

While lncRNAs and miRNAs have been extensively studied in the contexts of mesoderm and myogenic differentiation, a broader spectrum of non-coding RNA biotypes remains, which are still underexplored. SnoRNAs, tRNAs, and piRNAs are notable examples of these lesser-studied categories. The specific functions of these non-coding RNAs in mesoderm differentiation are largely unknown. Given their established roles in other cellular contexts, investigating sncRNAs could provide new insights into the molecular mechanisms governing mesodermal cell fate decisions and differentiation pathways with the potential of enhancing and improving myogenic differentiation and therefore reducing heterogeneity in current established differentiation protocols. Additionally, this line of research could also uncover novel regulatory networks and potential therapeutic targets for diseases associated with mesoderm-derived tissues, including muscle and cardiovascular disorders. This underscores the need for comprehensive studies to reveal the contributions of these diverse non-coding RNA types in mesoderm development.

Aim of Thesis

Despite significant advances in tissue engineering, several critical gaps remain, in particular, understanding the interplay between fundamental skeletal muscle biology and tissue engineering. One key area for improvement is the differentiation of myogenic cells from iPSCs, which holds promise for advancing this field. iPSCs are an ideal cell source due to their pluripotency, potential for patient-specific therapies, and unlimited self-renewal capacity. Their multilineage differentiation ability allows for greater complexity and better mimicking of in vivo environment, as previously demonstrated.[83,165] However, achieving efficient and consistent differentiation into fully functional myogenic cells remains a significant challenge.

Additionally, there is a critical need to explore and refine 3D models for skeletal muscle, as these models can more accurately mimic the native muscle environment. Such advanced models are essential not only for understanding muscle physiology and pathology but also for testing the functionality of engineered tissues. Improving functionality testing by developing new protocols and tools to assess therapy efficiency and efficacy is crucial. This dual exploration—integrating fundamental biology with innovative tissue engineering approaches—is essential for bridging existing gaps and driving innovation in skeletal muscle tissue engineering. By focusing on these areas, we aim to create more effective and precise models that closely resemble native tissue, ultimately improving therapeutic outcomes.

In Chapter 2, we investigate the identity of iPSCs and the role of non-coding RNAs during early germ layer differentiation to uncover their regulatory functions and impact on cellular differentiation processes, with implications for optimizing myogenic differentiation protocols. We also explore epigenetic memory in iPSCs and its influence on early germ layer differentiation to understand how prior epigenetic states affect differentiation and provide insights into optimizing reprogramming techniques and improving the fidelity of iPSC-derived cell types. This, in turn, can enhance myogenic differentiation and produce high-quality myogenic cells for 3D skeletal muscle engineering.

In Chapter 3, we study the development of an innovative magneto-conductive material for MEW to enhance 3D skeletal muscle modeling. We aim to identify and characterize materials that improve the structural and functional properties of engineered muscle tissues, facilitating more accurate and efficient skeletal muscle tissue fabrication.

In Chapter 4, we investigate various 3D skeletal muscle models for their utility in gene editing. By comparing different models, we aim to determine the most effective platforms for genetic modifications, potentially leading to improved treatments for muscle-related diseases.

In Chapter 5, we provide a detailed protocol for RNA and protein staining in skeletal muscle tissues and microtissues. The development of reliable and reproducible staining techniques is crucial for analyzing gene and protein expression in engineered tissues, contributing to our understanding of muscle biology and pathology.

In Chapter 6, we summarize the key findings of this thesis, highlighting advancements in iPSC biology, novel materials for tissue engineering, and comparisons of 3D models. The key findings are put in a broader context and future directions are discussed.

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