

**Investigating the cellular foundations of breast muscle myopathies in
broiler chickens**

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ABSTRACT

The genetic selection for high growth rate, breast muscle yield, and feed efficiency in modern broilers has been a double-edged sword. While it has resulted in the desired traits, it has also led to broiler myopathies. Recent omics studies have shown that hypoxia may be a potential factor that damages the myofiber niche and compromises muscle repair processes. The objective of this study was to optimize isolation protocols for primary broiler satellite cells (SCs) and fibroadipogenic progenitors (FAPs) and to characterize SCs proliferation and differentiation under hypoxia. The study first focused on the optimization of isolation procedures and culture conditions for primary broiler SCs. Simple and cost-effective preplating techniques were used to purify SCs, which may improve the fidelity of follow-on experiments. In addition, myogenic differentiation potential was maximized under optimal conditions with different types and concentrations of serum and chicken embryo extract. This dissertation further demonstrated the effects of hypoxia on the differentiation and metabolism of SCs. Satellite cells were exposed to hypoxia conditions for 48 h and normoxia recovery. Hypoxia improved SCs proliferation by maintaining Pax7 and reducing Myod1 and myogenin while reducing myogenic differentiation. The cell proliferation was not rescued by 24 h normoxia recovery, whereas myogenic potential was recovered. In addition, hypoxia-activated AMPK and upregulated proteins involved in lipid metabolism and cellular stress, with decreased mitochondrial activity and increased lipid accumulation in myotubes, which indicates that the balance between fatty acid uptake and oxidation was disrupted. Several differentially expressed genes were also identified across myogenic differentiation under hypoxia with targeted RNA sequencing. Lastly, we established an isolation protocol for broiler FAPs for the first time. Through the comparative analysis between FAPs and preadipocytes, the induction of adipogenic differentiation and morphology of adipocytes derived from muscle and adipose tissues are similar. However, fatty acid methyl ester and targeted RNA sequencing data revealed a distinct separation between FAPs and preadipocytes throughout adipogenic differentiation. In conclusion, these data provide valuable insight into the biology of SCs and FAPs and the effects of hypoxia on SC proliferation and differentiation to better understand broiler myopathies.

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INTRODUCTION

Literature review

Skeletal muscle development and growth are very important for human beings because it plays roles in regulating the protein metabolism of our body as well as glucose and lipid homeostasis. In addition, farm animals' skeletal muscle production is also vital because these are directly associated with on-farm benefits and global demand for protein sources. Specifically, the U.S. poultry industry has a significant impact on the economy, which provides about \$555.9 billion in economic activity (NCC., 2022). Thus, it is imperative to better understand the regulation of skeletal muscle development and growth.

Skeletal muscle characteristics

Skeletal muscle comprises 30-40% of total body mass and is essential for all body movements, and it is composed of multiple bundles of contractile muscle fibers (also called myofibers) that are covered by fascia (collagen-rich connective tissue layers) and surrounded by blood vessels and nerves. Myofibers are bundles of myofibrils that are made up of thin myofilaments (actin) and thick myofilaments (myosin), the two functional proteins required for muscle contraction and relaxation. Fascia provides structure to the fiber bundles, and blood vessels between muscle fibers play important roles in nutrient supply, oxygen delivery, and removal of cell metabolic waste and carbon dioxide. Skeletal muscle fibers are generally classified as slow-twitch or fast-twitch, based on their functional and metabolic roles. Slow-twitch fibers, compared to fast-twitch fibers, have a higher density of capillaries, myoglobin, and mitochondria that make them darker and reddish in color (Schiaffino and Reggiani, 2011). Slow-twitch fibers derive energy from oxidative metabolism and are capable of sustained rounds of contraction that are needed for endurance activities. Fast-twitch fibers rely on anaerobic metabolism (glycolysis), which provides acute bursts of energy needed for short-term and intense contractions. A variety of factors influence the relative proportions of myofibers with different metabolic properties, including genetic and physiological factors, nutrients, and hormones (Hocquette et al., 2010). For instance, a higher proportion of glycolytic myofibers is associated with genetic selection with rapid growth rates and leanness in animals (Hocquette, 2010). These muscle fibers are generated from muscle stem cells through successive waves of myogenesis.

Skeletal muscle development

Skeletal muscle development is the story of how/where muscle stem cells are generated and forms muscle fibers. In early embryonic development, a mesodermal layer is

developed during gastrulation, which takes part in forming musculoskeletal system, cardiovascular system, and connective tissues in the body. Skeletal muscle develops from the paraxial layer of the mesoderm, which is closest to the midline of the embryo. This layer gives rise to a population of stem cells that are committed to the skeletal muscle lineage. Paraxial mesoderm give rise to somites, and dorsal part of somites becomes dermomyotome (Kiefer and Hauschka et al., 2001; Bentzinger et al., 2012). The dermomyotome disintegrates into two layers, one of which forms the myotome, the first muscle mass that is formed (Tajbakhsh and Buckingham, 2000). Muscle stem cells in the myotome differentiate through a process known as myogenesis to form primary muscle fibers. Primary muscle fibers continue to grow in the embryo as muscle stem cells differentiate and fuse with existing muscle fibers. Then, another fusion of myoblast derived from muscle stem cells forms secondary fibers on the surface of the primary fibers that serve as a scaffold for the growth of fetal muscles (Biressi et al., 2007; Stockdale, 1992). The development of muscle fibers is almost finished at the time of birth/hatch. After this point, muscle growth results primarily from muscle fiber enlargement (hypertrophy), when adult muscle stem cells (known as satellite cells; SCs) undergo myogenesis and fuse with existing fibers (Velleman et al., 2007). The number of fused myonuclei is a determinant of muscle fiber size and strength (Gundersen and Bruusgaard, 2008).

Muscle stem cells

Muscle stem cells are derived from the dermomyotome and characterized by expression of Pax3 and Pax7. These two transcription factors maintain muscle stem cells in the undifferentiated state and support their ability to proliferate (Shirakawa et al., 2022) until the appropriate cues induce myogenesis. In mice, genetic deletion of Pax3/Pax7 prevents embryonic muscle development and induces cell apoptosis, which demonstrates the expression of Pax3/Pax7 of muscle stem cells is critical in skeletal muscle formation and development (Kassar-Duchossoy et al., 2005; Relaix et al., 2005).

Pax3 vs. Pax7 in muscle stem cells

Both Pax3 and Pax7, paired-box transcription factors, play important roles in developmental and adult regenerative myogenesis. Pax3 and Pax7 proteins are highly similar at the amino acid level and bind to the same DNA binding site and regulate expression of an overlapping set of genes (Goulding et al. 1991; Relaix et al., 2005; Soleimani et al., 2012). However, functional difference between Pax3 and Pax7 in myogenesis is observed.

Embryonic myoblasts are contributed from Pax3⁺ cells, whereas fetal myoblasts are originated from Pax7⁺ cells, which indicates Pax3 primarily influences embryonic development and Pax7 exerts its influence during perinatal growth (Hutcheson et al., 2009). Pax3 expression is downregulated before birth/hatch, while Pax7 is subsequently expressed in muscle stem cells from embryonic development. Pax7 deletion has no effect on embryonic development but causes significant loss of the satellite cell pool (Seale et al., 2000). Thus, both Pax3 and Pax7 play important roles in myogenesis but have nonredundant roles (Chang et al., 2014).

Myogenesis

Myogenesis describes the process through which muscle stem cells differentiate to produce contractile muscle fibers. Myogenesis results from sequential changes in gene expression that are orchestrated by a family of four basic helix-loop-helix (bHLH) transcription factors that are known as myogenic regulatory factors (MRFs), such as Myf5, MyoD, MRF4 (also known as Myf6), and myogenin (Rudnicki et al., 1993; Kassam-Duchossoy et al., 2005). The roles of MRFs are highly conserved across vertebrate species. Forced expression of any one of the MRFs can induce expression of muscle-specific genes in fibroblasts and other non-myogenic cell types, demonstrating their critical role in controlling myogenesis (Thayer et al., 1989). MRFs take part in muscle generation at multiple time points. In chicken, embryonic day 3-7, onset of Myf5 and MyoD protein expression marks the transition from stem cells into myoblasts that mainly undergo proliferation (Bentzinger et al., 2012). As Myf5 expression is increased, downregulation of Pax3 is initiated (Bober et al., 1994). Myoblasts continue to express Pax7 while proliferating through several cycles before exiting the cell cycle to differentiate further. Some myoblasts do not withdraw from cell cycle and go back to quiescence (reversible growth arrest) without differentiating. Those cells are defined as SCs regarded as adult muscle stem cells which can undergo myogenesis at the postnatal stage (Kuang and Rudnicki, 2008). Then, myoblasts exit the cell cycle and lose expression of Pax7, MyoD, and Myf5 and gain expression of MRF4 and myogenin, leading to differentiation into myocytes. Then, myocytes fuse with each other to form multinucleated primary muscle fibers.

Control of proliferation and differentiation in muscle stem cells

Muscle development involves a series of events that require signaling pathways and transcription factors to regulate myogenesis of muscle stem cells by regulating MRFs. For

example, Notch, Wnt/ β -catenin, bone morphogenic protein (BMP), and sonic hedgehog signals (shh) play pivotal roles in balance between proliferation and differentiation in muscle stem cells during embryonic development.

Notch signaling is known as a major regulator of muscle stem cell behavior and cell metabolism. In general, Notch signaling activates and mediates cell-cell interactions by engaging with transmembrane ligands such as Delta and Jagged ligands on neighboring cells. Activated Notch signaling by Delta1 can suppress the formation of muscle stem cell pool in the mesoderm, but muscle stem cells in dermomyotome can only differentiate into myogenic cells when the cells interact with Delta1-expressing cells (Kuroda et al., 1999; Conboy and Rando, 2002). Notch signaling maintains Pax7 expression, whereas downregulates MyoD in muscle stem cells. Forced activation of Notch signaling leads to an increased population of Pax7⁺/MyoD⁻ muscle stem cells and delays myogenic differentiation (Delfini et al., 2000). In addition, active Notch signaling represses MyoD expression through RBPJ (Notch transcriptional co-effector) and Hes/Hey family (Notch downstream genes) (Bentzinger et al., 2012). Conversely, deleting Delta1 or RBPJ induces MyoD expression, resulting in premature myogenic differentiation (Lahmann et al., 2021). RBPJ plays an important role in maintaining the population of muscle stem cells in a quiescent state and inhibiting myogenesis (Vasyutina et al., 2007). Ablation of oscillatory expression of Delta1 and Hes1 in muscle stem cells sustains MyoD expression (Lahmann et al., 2019). Notch signaling also plays important roles in producing extracellular matrix (ECM) collagens to keep muscle stem cells in quiescent state (Baghdadi et al., 2018).

Sonic hedgehog (Shh) is necessary to induce myotome formation. Deletion of Shh reduces expression of Myf5 and MyoD and increases the population of muscle stem cells in the somite while inhibiting a commitment to the myogenic lineage (Feng et al. 2006; Hammond et al. 2007). Ectopic Shh expression represses expression of Pax3 in dermomyotome (Borycki et al. 1998). This data indicates that Shh is required to reduce expression of Pax3 and induce expression of Myf5 and MyoD.

Along with Shh, Wnt/ β -catenin signaling is crucial in biological process, including cell attachment efficiency cell proliferation and cell fate decision (Klaus and Birchmeier, 2008). The canonical The Wnt pathway regulates target gene expression by binding Wnt to Frizzled family receptors, which induce intracellular accumulation of β -catenin, and subsequent translocation of β -catenin to the nucleus. Wnt1 directly activates Myf5 expression through the canonical β -catenin pathway, and Wnt7a or Wnt6 activates MyoD through a β -catenin-independent pathway (Borello et al., 2006; Brunelli et al., 2007).

Additionally, Wnt/ β -catenin signaling is necessary for dermomyotome development, and it increases the number of dermomyotomal muscle stem cells. (Hutcheson et al., 2009).

BMP signaling regulates the expression of target genes by mediating Smad which is a group of intracellular proteins. Upon binding of BMPs to transmembrane receptors, Smad transduces the signals from the cytoplasm to the nucleus (Miyazono et al. 2005). Bone morphogenic proteins (BMPs) have been known to inhibit myogenesis. BMP4 enhances Pax3 expression and delays the onset of Myf5 and MyoD expression (Pourquie et al. 1995). Moreover, activation of BMPs signaling increases the number of muscle stem cells (Wang et al., 2010). This indicates that BMP fosters the proliferation of muscle stem cells and tends to inhibit their differentiation to myogenic lineage.

Satellite cells – adult muscle stem cells

Satellite cells are a population of Pax7⁺ muscle stem cells that persist in adults to provide the capacity for muscle growth and repair after birth/hatch. Satellite cells are named for their location. They are located adjacent to muscle fibers between the basal lamina and the plasma membrane (Mauro, 1961). In mice, SCs are emerged 2 days before birth/hatch, proliferate until 12 days after birth/hatch, and remain in a quiescent state thereafter (Tajbakhsh, 2009). In chickens, they are emerged on embryonic day 14, and their population significantly increases at embryonic day 18 (Yablonka-Reuveni, 1995). Satellite cell proliferation occurs throughout embryonic days, but differentiation does not occur (Chal and Pourquié, 2017). Until 8 days after birth/hatch, chicken SCs remarkably proliferate and differentiate for muscle growth (Allouh et al., 2008; Halevy et al., 2020). Then, their activity declines, and they remain in a quiescent state. This cell population accounts for 1-5% of all nuclei in a healthy adult muscle fiber (Halevy et al., 2020).

Satellite cells are a heterogeneous population, including cells (Pax7⁺/Myf5⁻) that are capable of self-renewal and cells (Pax7⁺/Myf5⁺) that are committed to myogenesis. The subpopulation that is capable of self-renewal provides a persistent supply of stem cells that are used during muscle repair. When SCs are activated, they replicate themselves (self-renew) in order to produce more stem cells through symmetric or asymmetric division. Because of this property, SCs are called adult muscle stem cells. When they divide in an asymmetric manner, a daughter progenitor cell undergoes myogenic differentiation, and the other daughter stem cell replenishes the muscle cell reservoir. Symmetric divisions generate two identical self-renewing, differentiating, or undetermined daughter cells (Collins et al., 2005; Shinin et al., 2006; Kuang et al., 2007; Brack and Rando, 2012; Tierney and Sacco,

2016). The division of SCs is regulated depending on normal or pathological conditions (Kuang et al., 2007). For example, in healthy muscles, satellite stem cells do not need to expand and differentiate. Thus, the asymmetric division is efficient for maintaining muscle stem cell pools for future use. However, in response to acute injuries, they need to rapidly expand for effective regeneration; thus, symmetric division is appropriate to produce enough myonuclei (Kuang et al., 2008).

Interestingly, several studies showed that SCs can transdifferentiate into chondrogenic, fibrogenic, or adipogenic lineage under specific conditions, although those fates are not documented to occur in vivo (Asakura et al., 2001; Brack et al., 2007; Yin et al., 2013). Shefer et al. (2004) reports that adipogenic SCs expressed both Pax7 and adipogenic markers, PPAR and C/EBP.

Regulation of satellite cell quiescence, proliferation, and differentiation

Under homeostatic environments, SCs remain in a quiescent state. In response to muscle injuries, physical exercise, or inflammation, SCs are activated and induced to proliferate. After several cycles of proliferation, SCs differentiate to provide sources of myonuclei to the damaged fibers or to form new myotubes. At the same time, a subset of SCs returns to a reversible quiescence state to regenerate the satellite cell pool for future use. These processes are controlled by several transcription factors. Of these, Pax7 is indispensable in satellite cell function as it regulates satellite cell proliferation and differentiation as well as survival. Pax7 is highly expressed in quiescent and proliferating SCs (Olguin and Olwin, 2004; Zammit et al., 2006; Lepper et al., 2011). When Pax7 expression is suppressed, it initiates the terminal myogenic program of SCs, while maintaining its expression keeps them in a quiescent state or enhances their proliferation (Soleimani et al., 2012). Pax7^{high} SCs tend to have a higher self-renewal capacity and a lower myogenin expression than Pax7^{low} satellite cells (Rocheteau et al., 2012).

The regulation of Pax7 is complex and involves environmental factors, nutrition, signaling pathways, and epigenetic modifications. Understanding the mechanisms that regulate Pax7 expression and function is important for attenuating muscle disorders and injuries. Under homeostatic environments, SCs remain in a quiescent state. Environmental factors, such as physical exercise, can transiently decrease Pax7⁺ SCs, but eventually, increase Pax7⁺ SCs by promoting satellite cell activation and proliferation (Chen et al., 2020; Masschelein et al., 2020). In addition, heat stress and low oxygen level increase the number of Pax7⁺ SCs (Kojima et al., 2007; Liu et al., 2012). Along with exercise, nutritional factors,

such as Vitamin D and omega-3 fatty acids (EPA and DHA), can also increase/decrease Pax7 expression (Hutton et al., 2014; Hsueh et al., 2018; Cheung et al., 2020). Several studies have shown that Notch signaling can regulate the expression of Pax7 in SCs. When Notch signaling is activated, it can promote the proliferation of SCs by upregulating Pax7 expression. In contrast, when Notch signaling is inhibited, SCs tend to differentiate and Pax7 expression decreases (Wen et al., 2012; Pasut et al., 2016). Epigenetic modifications, such as DNA methylation and histone modifications, also play a role in regulating Pax7 expression. The modification can be caused by acetyltransferase MYST1, histone deacetylase HDAC4, histone methyltransferase H3K4me3, and arginine methyltransferase CARM1 (Addicks et al., 2019; Sincennes et al., 2021). For instance, loss of mixed lineage leukemia protein-1 in SCs reduces histone methyltransferase (H3K4me3) enrichment at both Pax7 and Myf5 loci related to the loss of Pax7 and Myf5 expression, resulting in decreased satellite cell proliferation and self-renewal (Kawabe et al., 2012; Addicks et al., 2019). Ubiquitin-proteasome system plays a role in regulating Pax7 levels and activity (Kitajima et al., 2018; Olguin et al., 2022). Casein kinase 2 phosphorylates Pax7, which inhibits Pax7 degradation by caspase-3 in proliferating muscle progenitors (Olguin et al., 2022).

Several studies have reported that energy metabolism plays a critical role in regulating satellite cell fate decision, including, quiescence, activation, proliferation, differentiation, and self-renewal (Ryall, 2013; Tang and Rando, 2014; Ryall et al., 2015; Pala et al., 2018; Yucel et al., 2019). Quiescent SCs have a reduced metabolic rate due to their low energy demand, which helps to maintain their quiescent state and prevent them from undergoing differentiation (Fiacco et al., 2016; White et al., 2018). Quiescent SCs primarily rely on fatty acid oxidation for energy (Ryall et al., 2015; Yucel et al., 2019). However, upon activation, SCs undergo a metabolic shift towards increased glucose uptake and glycolysis, providing the necessary energy and biosynthetic precursors (nucleotides, lipids, and proteins) for cell proliferation and differentiation (Rocheteau et al., 2012; Rodgers et al., 2014; Dell'Orso et al., 2019). Differentiating SCs are characterized by predominant oxidative phosphorylation and fatty acid oxidation, along with increased mitochondrial biogenesis (L'honoré et al., 2018). This leads to increased production of mitochondrial reactive oxygen species (ROS), which act as secondary messengers to induce myogenesis. Treatment of N-acetylcysteine, an inhibitor of ROS, reduces myogenesis but increases proliferation (L'honoré et al., 2018).

Recently, 5'-AMP-activated protein kinase (AMPK) has been recognized as an important regulator of satellite cell fate, especially self-renewal. Self-renewal of SCs is a

process by which activated SCs reversely return to quiescence SCs to maintain stem cell pool (Kuang et al., 2008). AMPK is an important cellular energy sensor that plays a crucial role in regulating cellular energy metabolism (Hardie et al., 2012). It is activated in response to low energy status, such as during exercise or calorie restriction, and promotes glucose uptake and lipid oxidation that generate ATP, the primary source of energy in cells (Long et al., 2006). Fu et al. (2015) reported that AMPK is required for satellite cell proliferation and differentiation. In the study, AMPK deficiency impairs activation and proliferation by inhibiting Warburg-like glycolysis. However, AMPK α 1 deficiency doesn't have negative impact on the maintenance of satellite cell quiescence (Theret et al., 2017). However, in injured muscles, AMPK α 1-deficient SCs utilize more glycolytic metabolism and display enhanced self-renewal. Along with AMPK, liver kinase b1, an upstream activator of AMPK, also plays an important role in maintaining a quiescent state. Liver kinase b1 mediates AMPK to inhibit the mTOR signaling that induces cell cycle entry of quiescent stem cells from a G0 to a G1, resulting in activating SCs (Rodgers et al., 2014; Shan et al., 2014).

Skeletal muscle regeneration

Muscle damage can be triggered from exercise, physical injury, stress, aging, or diseases in daily life. Skeletal muscle regeneration is a coordinated process involving cell-cell and cell-ECM interactions between SCs, inflammatory cells, and FAPs to restore muscle tissue homeostasis sequentially. This process involves key events such as removal of damaged muscle fibers, transient collagen deposition, proliferation/differentiation of SCs, regeneration of muscle fibers, followed by connective tissue remodeling. As the muscle capillary system is damaged immediately after injury, the capillary network also is remodeled through upregulation of angiogenic genes, activation of endothelial cells, and regeneration of microvessels (Hardy et al., 2016; Fernando et al., 2019). This regeneration process is supported by growth factors and cytokines released from damaged blood vessels, degraded ECM, and inflammatory cells (Goetsch et al., 2003; Chazaud et al., 2009).

To be specific, after injury, Neutrophils first invade the injury site, and their number rapidly increases to evoke the inflammation response (McClung et al., 2007). Infiltrating pro-inflammatory macrophages then remove debris of dead or damaged myofibers by phagocytosis, releasing pro-inflammatory cytokines, such as TNF- α and IL-6 is released. In addition, they recruit and activate SCs. SCs proliferate and differentiate to form new myotubes, which in turn ultimately generate new myofibers (Charge et al., 2004; Sacco et al., 2008). At the same time, FAPs proliferate and promote the expansion of SCs (Joe et al.,

2010; Uezumi et al., 2010). Additionally, FAPs are activated to produce ECM proteins for ECM remodeling (deposition and degradation) which is necessary for new muscle fiber formation by SCs (Arnold et al., 2007; Wynn et al., 2008). Then, FAPs begin to undergo apoptosis due to the TNF- α secreted from pro-inflammatory macrophages (Lemos et al., 2015). Inflammation is resolved as pro-inflammatory macrophages are replaced by anti-inflammatory macrophages. The population of anti-inflammatory macrophages is increased, and secreted anti-inflammatory cytokines and TGF- β . This enhances satellite cell differentiation and supports the survival of remaining FAPs.

Aberrant muscle repair

The process of healthy muscle repair is disrupted in various muscle diseases, and can be affected by chronic inflammation, aging, and genetic mutations. When this occurs, the repaired lesions are infiltrated with fibrofatty tissue, which is produced by aberrant differentiation of FAPs into adipocytes and fibroblasts, rather than healthy myofibers (Joe et al., 2010; Uezumi et al., 2010). This compromises muscle strength and function. In addition, reparative capacity of SCs is reduced.

Prolonged inflammation in the affected muscle can disrupt the normal repair process and lead to the formation of fibrofatty tissue instead of healthy muscle fibers. To be specific, anti-inflammatory macrophages release TGF- β to activate FAPs to produce ECM proteins during muscle regeneration. However, under chronic inflammation, the persistent presence of anti-inflammatory macrophages results in the secretion of more anti-inflammatory cytokines and TGF- β , which supports the survival of remaining FAPs that are supposed to undergo apoptosis. This promotes fibroblasts transition, leading to excessive ECM accumulation and fibrosis (Contreras et al., 2019).

Aging leads to aberrant muscle repair by loss of satellite cell pool and regenerative potential (Sousa-Victor et al., 2022). Numerous studies found that ageing is likely to promote activation, differentiation, and senescence rather than quiescence (Yin et al., 2013; Baghdadi et al., 2018). For instance, fibroblast growth factor-2 production is increased by aged fibers, leading to reduced satellite cell quiescence and prolonged activation. This could potentially lead to depletion of satellite cell pool. Besides, aging also influence proliferation, differentiation, and function of FAPs. The number of FAPs isolated from aged muscles is reduced, and proliferation ability is decreased in aged FAPs. Remarkably, aged FAPs exhibit reduced adipogenic potential and induced fibrogenic potential compared to young FAPs (Mueller et al., 2016; Lukjanenko et al., 2019). In addition, aged FAPs displays impaired

function, including reduced production of WNT1-inducible signaling pathway protein 1 (WISP1) that plays important roles in controlling the expansion and asymmetric commitment of SCs.

Along with inflammation and aging, genetic mutations can also impact functions of SCs. For instance, lack of dystrophin leads to prolonged satellite cell activation and differentiation, resulting in depletion of satellite cell pool (Blake et al., 2002). Mutations in the dystrophin gene can lead to the development of muscular dystrophies.

Muscle myopathies

Myopathies are a class of neuromuscular disorders that are characterized by muscle weakness, stiffness, and loss. The loss of satellite cell capacity to proliferate and differentiate, as well as an exhausted satellite cell pool, are common features of myopathies (Mann et al., 2011). In addition, newly generated muscle fibers particularly following injury or disease, are vulnerable to degeneration due to molecular defects, such as misfolded proteins (Porter et al., 2002). Representative myopathies are Duchenne muscular dystrophy (DMD) and age-related sarcopenia.

DMD is a muscle-wasting disease characterized by progressive muscle degeneration and necrosis due to the mutations in dystrophin that help keep muscle cells intact. This gene is on the X chromosome, resulting in that DMD carriers are females (Duan et al., 2021). The majority (60–70%) of mutations in DMD patients are deletions, followed by duplications (5–15%), point mutations (20%), and minor deletions or insertions (10%) (Magri et al., 2011). It is found that damaged muscles from patients and animal models with DMD generate more free radicals than normal muscles. The increased free radical is mostly derived from NADPH oxidase 2-mediated reactive oxygen species production (Khairallah et al., 2011), resulting from infiltrating inflammatory cells and dysfunctional mitochondria (Grounds et al., 2020). In addition, DMD muscle is susceptible to oxidative stress due to reduced levels of glutathione, an important antioxidant (Dudley et al., 2006). Higher concentrations of Ca^{2+} are maintained in DMD muscle compared to that in healthy muscle, which can induce mitochondrial dysfunction (Millay et al., 2008).

Age-related sarcopenia exhibits loss of muscle mass, muscle weakness, and muscle dysfunction. It mainly occurs in older people (Age < 60) and highly affects people with diabetes. In people aged ~75 y, muscle mass declines at a rate of 0.8-0.98%/y in males and 0.64-0.7%/y in women. Male and female muscle strength declines at rates of 3–4%/y and 2.5–3%/y, respectively. (Mitchell et al., 2012). Sarcopenia is characterized by diminished

mitochondrial respiratory activity, contractile dysfunction, elevated oxidative stress, reduced satellite cell count, senescence phenotype, loss of motor units, neuromuscular junction degradation, dysregulated calcium homeostasis, and hormonal changes (Parvatiyar et al., 2022). Age-related sarcopenia may result from extended quiescence of SCs, resulting in the accumulation of misfolded proteins, mitochondrial damage, ROS, and DNA damage (Rodgers et al., 2014; Zismanov et al., 2015; Machado et al., 2016). Thus, the absence of periodic activation and asymmetric division may have a deleterious impact on long-term viability, impairing regenerative capacity (Chen et al., 2020).

Fibroadipogenic precursors – non-myogenic cells that support muscle repair

Since their discovery by both Mauro and Katz in 1961, SCs have been the focus of studies related to muscle growth, repair, damage, and disease. More recently, an additional muscle stem cell population, known as FAPs, have been shown to play an important role in muscle homeostasis. Fibroadipogenic progenitors are interstitial muscle-resident mesenchymal stem cells that are named because of their ability to differentiate into both fibroblasts and adipocytes. The importance of this novel cell type in both healthy muscle regeneration and myopathies was first highlighted in 2010 by two independent groups studying the basis for muscular dystrophies in humans (Joe et al., 2010; Uezumi et al., 2010). These studies demonstrated that FAPs crosstalk with SCs, and that these interactions play an important role in supporting satellite cell growth and differentiation in response to muscle injury. Moreover, disruption of this crosstalk was shown to result in aberrant differentiation of FAPs to fibroblasts and adipocytes, and ultimately to formation of the fatty and fibrotic lesions that characterize myopathies and other muscle diseases FAPs (Uezumi et al., 2011; Mozzetta et al., 2013; Kopinke et al., 2017).

Features of fibroadipogenic progenitors

Fibroadipogenic progenitors exhibit properties of mesenchymal stem cells and can differentiate into adipocytes, fibroblasts, osteoblasts, and chondrocytes (Wosczyzna et al., 2012; Marinkovic et al., 2019; Eisner et al., 2020; Giuliani et al., 2021), although the latter two fates are not documented to occur in vivo. Fibroadipogenic progenitors are characterized by the surface expression of platelet-derived growth factor receptor alpha (PDGFR α), a membrane-bound tyrosine kinase receptor (Contreras et al., 2021). Signaling through PDGF receptor pathways regulates angiogenesis, stem cell fate decision, and proliferation in multiple tissues. Fibroadipogenic progenitors are relatively abundant in skeletal muscle, as

PDGFR α ⁺ cells account for 5–15% of the total nuclei and 20–30% of the interstitial mononuclear cells in adult muscles (Contreras et al., 2021). Fibroadipogenic progenitors produces ECM proteins, such as collagen, metalloproteinases, fibronectin, laminin, and proteoglycans, providing a favorable niche for the self-renewal of SCs as cellular sources of ECM (Rayagiri et al., 2018; Lukjanenko et al., 2019).

Crosstalk between fibroadipogenic progenitors and satellite cells

In addition to providing components of the ECM, FAPs play a pivotal role in maintaining muscle homeostasis and supporting muscle repair by crosstalk with SCs and immune cells. Depletion of FAPs can trigger muscle atrophy, with decreased muscle mass, fiber size, and satellite cell pool, alluding that FAPs are required to maintain the satellite cell pool and to enhance the proliferation/differentiation of SCs (Petrilli et al., 2020). In healthy muscles, FAPs and SCs are normally quiescent. Upon muscle damage, both populations are activated and enter the cell cycle, with FAPs expansion occurring before satellite cell expansion. FAPs secrete trophic factors that support satellite cell differentiation. This is supported by the fact that the presence of FAPs downregulates the expression of Pax3 and Pax7 in SCs while upregulating the expression of MyoD and myogenin (Joe et al., 2010). Moreover, myogenesis is regulated by paracrine signals released from FAPs, such as IL-6, IL-10, IL-33, follistatin, BMP3, and Wnt family member 1 (Joe et al., 2010; Fiore et al., 2016; Biferali et al., 2019; Wosczyzna et al., 2019). As muscle regeneration proceeds, FAPs produced to support repair are induced to undergo apoptosis, and rapidly goes back to their original number (Natarajan et al., 2010; Lemos et al., 2015). Failure of this sequence of communication between FAPs and SCs or in the apoptotic clearance of FAPs results in their inappropriate differentiation to fibroblasts and adipocytes (Lemos et al., 2015; Buras et al., 2019; Hogarth et al., 2022).

Factors that regulate the fate of fibroadipogenic progenitors

The aberrant differentiation of FAPs to fibroblasts or adipocytes is thought to be induced by cytokines and metabolites released by SCs, myotubes, inflammatory cells, and FAPs themselves (Biferali et al., 2019; Moratal et al., 2019). For instance, FAPs proliferation is enhanced by growth factor released from SCs. In addition, myoblasts and myotubes-derived conditioned medium inhibits adipogenesis of FAPs and induce gene expression involved in fibrogenesis, indicating that factors released from terminal myogenic differentiation can regulate FAPs fate decision and production of ECM proteins (Murphy et

al., 2011; Fiore et al., 2016). When FAPs derived from cardiotoxin-injured muscles (no adipogenesis) are transplanted into glycerol-injured muscles (adipogenesis), FAPs differentiates into adipocytes. In the opposite case, FAPs failed to differentiate into adipocytes (Joe et al., 2010; Uezumi et al., 2010; Pagano et al., 2019). Altogether, these data suggest that the fate decision of FAPs is crucial for healthy muscles and tightly controlled by environmental signals.

Among these secreted factors, TGF- β , PDGF-AA, myostatin, and wnt3A have been shown to induce fibroblastic differentiation of FAPs. TGF- β is known as a hallmark of fibrosis and is a secreted protein of macrophages, FAPs, and regenerating myofibers in skeletal muscle. TGF- β promotes fibrogenic differentiation of FAPs (Klingberg et al., 2014; Contreras et al., 2019; Lodyga et al., 2020). It has also been shown to promote proliferation and inhibit adipogenic differentiation (Contreras et al., 2019). TGF- β leads to the activation of canonical Wnt/ β -catenin signaling pathway, and nuclear translocation of β -catenin suppresses FAPs adipogenesis (Akhmetshina et al., 2012; Reggio et al., 2020). Suppression of the TGF- β signaling pathway reduces FAPs accumulation as well as fibrofatty tissue infiltration by enhancing FAPs apoptosis in damaged muscles (Davies et al., 2016).

PDGF signaling pathways are involved in angiogenesis, stem cell fate decision, and proliferation. PDGF ligands are secreted from macrophages and fibroblasts. They bind to PDGFRs, a membrane-bound tyrosine kinase receptor (Contreras et al., 2021). FAPs generate various transcriptional variants of PDGFR α through intronic polyadenylation. Intronic polyadenylation produces mRNA which encodes a C-terminal truncated protein (Elton et al., 2022). PDGFR α isoform encoded from one of variants contain a truncated kinase domain that acts as a decoy receptor of PDGF ligands. This inhibits PDGF signaling pathway in FAPs, leading to decreased ECM deposition and fibrosis. Conversely, downregulating intronic polyadenylation of PDGFR α stimulate FAPs activation and fibrosis (Muller et al., 2016). Treatment of ex vivo PDGF activates PDGF signaling pathway in FAPs, inducing FAPs activation and proliferation as well as gene expression involved in ECM (Contreras et al., 2019).

Along with TGF- β and PDGF signaling, retinoic acid, Notch, and cilia signaling have also been found to regulate FAPs expansion and differentiation. Retinoic acid treatment in FAPs enhances proliferation and suppresses adipogenesis and fibrogenesis (Zhao et al., 2020). Conversely, as retinoic acid receptor signaling is blocked, FAPs adipogenesis is promoted, and FAPs clearance by apoptosis is decreased. Notch signaling is a pivotal pathway regulating FAPs differentiation. Marinkovic et al. (2019) reported Delta1 secreted

from SCs and myofibers activates Notch signaling, inhibiting adipogenesis of FAPs. Interestingly, FAPs isolated from dystrophic muscle is unresponsive to Notch-mediated adipogenic inhibition compared with acute injury-derived FAPs. Cilia is a membrane-bound organelle that transduces hedgehog signals into FAPs. Ciliary hedgehog signaling is involved in initiating myogenic program during muscle development (Hu et al., 2012). Kopinke et al. (2017) reported that the intramuscular fat deposition is controlled by the ciliary hedgehog signaling in FAPs. Removing FAPs cilia inhibits adipogenesis by repressing Hedgehog signaling in glycerol-induced muscle injury, resulting in enhanced muscle regeneration. Therefore, the fate of FAPs is tightly regulated by multiple signaling pathways, and understanding these pathways may be important for healthy muscle regeneration and repair.

Fibrogenesis and adipogenesis of fibroadipogenic progenitors can influence satellite cell behavior

Proper coordination between the proliferation and differentiation of SCs and FAPs is required for healthy muscle repair. In pathological conditions, FAPs undergo prolonged expansion and differentiation into fibroblasts or adipocytes, causing excessive fibrofatty infiltration (Lemos et al., 2015; Pagano et al., 2015; Dammone et al., 2018; Madaro et al., 2018). Some studies show that intramuscular fat accumulation and fibrosis decrease insulin sensitivity of skeletal muscle in aged and obese mice (Kang et al., 2011; Sachs et al., 2019). In support of this notion, a co-culture system using adipocytes and muscle cells shows that adipocytes inhibit myogenesis of muscle cells and induce insulin resistance of muscle cells and myotubes derived from muscle cells (Kudoh et al., 2018; Seo et al., 2019). Additionally, the co-culture system increases adipogenic gene expressions and intracellular lipid deposition in muscle cells, leading to impaired myogenesis (Choi et al., 2013; Guo et al., 2018).

Fibrogenesis of FAPs can also influence satellite cell behavior. Fibrogenesis refers to the process by which FAPs differentiate into fibroblasts, which are then activated and produce excessive ECM in response to injury or chronic inflammation. Fibroblasts promote myoblast proliferation and enhance self-renewal while inhibiting differentiation (Murphy et al., 2011). However, under chronically injured conditions, activated fibroblast can produce excessive fibrotic ECM leading to ECM stiffness, which can negatively influence satellite cell proliferation and differentiation (Zhang et al., 2021). Under chronic injury, fibroblasts can be converted into myofibroblasts that contribute to the development and progression of

fibrosis (Loomis et al., 2022). They interact with SCs and influence satellite cell behavior. Satellite cell proliferation is increased when the number of myofibroblasts is limited (Thooyamani et al., 2021). In injured muscles, premature depletion of myofibroblasts leads to premature differentiation of SCs and early depletion of the satellite cell pool (Murphy et al., 2011). In addition, fibrogenesis and adipogenesis can lead to the accumulation of fibrofatty tissue within muscle, which can impede blood flow and limit the delivery of oxygen and nutrients to cells (Henke et al., 2020). This might further impair their ability to proliferate and differentiate. Overall, fibrogenesis and adipogenesis of FAPs can influence satellite cell proliferation and differentiation.

Myopathies are prevalent in the breast muscle of broiler chickens

In the last 50 years, the genetic selection of commercial broilers has been focused on high feed efficiency, larger breast muscle yield, and accelerated growth rate. As a result, modern broilers require less feed and a shorter amount of time to reach heavier market weight than their 1970s counterparts (NCC, 2022). More specifically, breast muscle weight has increased dramatically and now accounts for up to 25% of body weight (Scheuermann et al., 2003; Aviagen, 2019). Despite these benefits for broiler production, rapid improvements have come at a cost to the quality of the breast muscle. Broilers are now very vulnerable to several lesions in the breast muscle, collectively referred to as breast muscle myopathies. Different types of myopathies have been described and named based on various features of lesions, including wooden breast, white striping, deep pectoral myopathy, and spaghetti meat. Each alters the biochemical and morphological properties of skeletal muscle and compromises meat quality in ways that impose significant losses to the broiler industry. Lesions negatively affect the appearance and texture of broiler meat and discourage consumer acceptance, and lesioned filets are downgraded or discarded. Myopathy lesions are detected manually or visually when breast filets are processed at slaughter, so the costs of raising birds to market age are incurred before the defects are detected. The incidence of lesions in broilers is widespread, with estimates of incidence in commercial flocks as high as 90% (Tijare et al., 2016). Lesions are not restricted to a specific broiler line and occur across the globe. The US broiler industry alone has been estimated to lose ~ \$1B due to these myopathies (Barbut et al., 2019).

Wooden breast and white striping are the two most prevalent forms of myopathies in common commercial lines of broilers. Wooden breast lesions result when areas of the breast muscle (pectoralis major) become hardened and thickened, with hemorrhagic areas

on the surface of the muscle. White striping describes white fibrotic, lipid-rich striations on the surface of the pectoralis major. These two types of lesions are interrelated and are often found together in the broiler pectoralis major muscle (Aguirre et al., 2020). Even though it is not identified whether both white striping and wooden breast are caused by a common etiology (Velleman, 2015), they share many histological features. Histopathologically, both types of lesions in birds at slaughter age (~ 42 days) are characterized by signs of muscle fiber damage, inflammation, and fibrosis. Changes include muscle cell growth failure, ectopic deposition of adipose tissue, immune cell infiltration, myofiber necrosis, degenerative fibers, accumulation of interstitial connective tissue and collagen, and severe fibrosis (Papah et al., 2017; Velleman. 2019). Numerous transcriptomic studies have described widespread differences in gene expression between muscles of affected and non-affected broilers (Zambonelli et al., 2016; Pampouille et al., 2019; Bordini et al., 2022). As would be expected from the visible features of lesions, these studies have consistently shown upregulation of genes related to adipogenesis, lipid accumulation, inflammation, and fibrosis in muscle from affected birds.

Incidence and severity are related to rapid growth

The incidence of wooden breast and white striping is closely related to high feed efficiency and rapid growth (Mutryn et al., 2015), with heavier and fast-growing chickens most likely to develop lesions. While genetic selection led to the incidence of myopathies, evidence suggests that they did not arise due to direct effects of genetic variants that were introduced during selection. Estimates of heritability for incidence and severity of myopathies are very low (Lake et al., 2021), and there is little genetic correlation between incidence and body weight or breast yield. Rather, lesions are thought to result from the shared physiology of rapid breast muscle growth rather than from heritable genetic mutations (Bailey et al., 2015). The severity of lesions increases in parallel with the weight of the pectoralis major muscle and breast yield (Griffin et al., 2018). Intriguingly, wooden breast and white striping primarily affect breast muscle (specifically p. major), which has been the target of selection programs, and no other muscles, such as the leg, thigh, and wing to which growth rate has not been subjected to selection pressure. Pampouille et al. (2019) reported that fibrosis and adiposis are more observed in endomysium and perimysium of fast-growing chickens compared to slow-growing chickens.

Consequences of rapid growth that may damage muscle

The exact pathomechanisms of wooden breast, white striping, and other myopathies are still unknown, but their relationship to breast muscle yield suggests that physiological consequences of high feed efficiency and extremely rapid growth contribute to negative consequences on the muscle environment. Several cases of the incidence of myopathies have been proposed, such as deficiency of antioxidants, alterations in lipid and glucose metabolism, and hypoxia (Bordini et al., 2022; Malila et al., 2022; Velleman et al., 2022). A common feature of different proposed mechanisms of myopathies is that they begin with damage to myofibers and that the normal process of healthy muscle repair is impaired, resulting in fibrotic and fatty tissue in place of muscle fibers. A prevailing hypothesis is that myofibers are damaged due to an imbalance muscle growth and vascularization, and that the circulatory system cannot sufficiently supply oxygen and nutrients and remove metabolic wastes in pectoralis major due to its rapid rate of growth. A relative deficit in capillary density relative to myofiber area in pectoralis major has been described as an early feature of wooden breast, suggesting that reduced blood supply to myofibers precedes the development of focal lesions at 2 weeks of age (Sihvo et al., 2018). Reduced vascularization compared to the needs of the muscle compromises the ability of the circulatory system to effectively deliver oxygen and nutrients and remove waste products from muscle fibers. In addition to a deficit in oxygen and energy supply, insufficient vessel density would impair the removal of metabolic wastes such as lactic acid that can damage myofibers if not removed (Velleman et al., 2010). These structural changes are thought to further cause localized hypoxia within myofibers that induces inflammation and oxidative stress production, which eventually affect skeletal muscle homeostasis (Mutryn et al., 2015).

Satellite cell exhaustion and breast muscle myopathies

Commercial broilers are characterized by an extremely fast growth rate, which leads to excessive myofiber hypertrophic growth. These traits are the result of growth selection that occurs in the post-hatching period, during which SCs mainly drive muscle growth (Velleman et al., 2003). Unfortunately, myofiber hypertrophic growth reduces connective tissue spacing, which is required for the circulatory supply that satellite cell activity relies on, impairing satellite cell-mediated muscle regeneration (Velleman et al., 2022). As evidence of this, satellite cell-mediated muscle regeneration is slower in larger-size broilers compared to their smaller counterparts, along with diminished numbers of SCs and decreased abilities to proliferate and differentiate (Daughtry et al., 2017). This suggests that

strong genetic selection for broiler breast muscle growth may lead to the exhaustion of satellite cell pools.

Wooden breast-affected muscle exhibits decreased expressions of Pax7 and Myf5 and increased expression of MyoD and myogenin (Velleman and Clark, 2015; Emami et al., 2021). This alludes that SCs are likely used for myogenic differentiation for muscle regeneration rather than replenishing the satellite cell pool in wooden breast-affected muscles. Different studies show that there is a difference in the heterogeneity of SCs in severely affected breast muscles compared to unaffected muscles (Meloche et al., 2018; Ferreira et al., 2020). Specifically, in the total number of myogenic cells, the wooden breast-affected muscles have a higher percentage of Pax7⁺ cells compared to unaffected muscles, which are quiescent SCs that are important for maintaining muscle stem cell populations, and a lower percentage of Pax7⁺/MyoD⁺ cells, which are proliferating SCs that are involved in muscle regeneration and growth (Ferreira et al., 2020). Besides, the total number of myogenic cells is higher in wooden breast-affected muscles than in unaffected muscles (Meloche et al., 2018). This suggests that there is an impaired myogenic program in SCs that could lead to decreased satellite cell-mediated muscle regeneration in severely affected breast muscles. However, further research is needed to understand the underlying mechanisms and implications of these differences in myogenic cell heterogeneity between unaffected and affected muscles.

Early changes during development of myopathies in broiler breast muscle

One of the major challenges in understanding the basis for wooden breast and other myopathies is that incidence is not detected until slaughter when breast muscle is handled during processing. Therefore, the causative factors that lead to massive lesions in market-age birds are difficult to identify. Papah et al., (2017) conducted a novel prospective study in which they used a needle biopsy technique to sample breast muscle tissue over time in a large number of birds from one week of age to market age. They identified which birds went on to develop lesions at slaughter and then compared changes in histology and gene expression over time between birds with and without wooden breasts. They found that localized lipogranulomas and lipid infiltration between myofibers were detectable as early as one week of age in birds that eventually developed wooden breast lesions. By 2-3 weeks of age affected birds showed signs of single myofiber degeneration and inflammation that progressed to fiber degeneration, necrosis, and fibrosis from week 4 to week 7 (Papah et al., 2017). White striping, which includes ectopic deposition of lipid and adipocytes in breast

muscle, is reported as the initial step of the ontogeny of wooden breast and is visibly detected as early as 16 days of age before wooden breast lesions are detected (Griffin et al., 2018). This group also used biopsy samples to identify changes in gene expression that occurred early during the development of muscle lesions. Birds that went on to develop wooden breast demonstrated increased expression of genes involved in lipid metabolism as early as 2 weeks of age. Expression of the fatty acid transporters, fatty acid-binding protein 4, and a cluster of differentiation 36 was higher in the muscle that ultimately developed wooden breast lesions. Also, perilipin 2, fatty acid synthase, lipoprotein lipase, CCAAT enhancer-binding protein alpha, and peroxisome proliferator-activated receptor-gamma may contribute to the higher adiposis observed in fast-growing chickens plagued with white striping and wooden breast (Papah et al., 2018; Lake et al., 2019; Pampouille et al., 2019). Given these features, lipid infiltration is considered an initial symptom, and it is considered necessary to investigate where and how ectopic adipose tissues are aroused to uncover the origin of the disease.

Fibroadipogenic precursors – a contributor to myopathies in broilers?

Fibroadipogenic progenitors have clearly been established to play a role in the formation of myopathies in humans. However, to our knowledge, FAPs have not yet been characterized in chickens, and their relationship to broiler myopathies has not been well investigated yet. Previous studies have shown that FAPs may be involved in the development of wooden breast in chickens. Reuveni et al. (1988) demonstrated that non-myogenic cells in breast muscle possess binding sites for PDGF, and PDGFR α is upregulated in affected muscles (Papah et al., 2018; Pampouille et al., 2019; Praud et al., 2020). Moreover, aberrant differentiation of FAPs could contribute to excessive fibrofatty tissue infiltration in wooden breast-affected muscle lesions. However, further research is needed to fully understand the role of FAPs in the development of wooden breast.

Evidence that hypoxia contributes to development of myopathies in broilers

Hypoxia is a key characteristic of broiler myopathies, resulting from a relative deficit in capillary density relative to myofiber area in pectoralis major. It is evident that localized tissue hypoxia dysregulates oxygen homeostasis in circulatory and breast muscles, resulting in the disruption of muscle homeostasis (Mutryn et al., 2015). In addition, broiler myopathies exhibit localized tissue hypoxia with a higher partial pressure of carbon dioxide (pCO₂), lower partial pressure of oxygen (pO₂), and lower oxygen saturation (sO₂) (Lake et

al., 2020). This hypothesis is supported by a study showing changes in oxygen transport-related molecules as well as low oxygen and hemoglobin levels in broilers affected by wooden breast compared to healthy counterparts (Greene et al., 2019). Elevated expression of hypoxia-inducible factor 1 alpha (HIF-1 α), a transcription factor that senses cellular hypoxia, is detected early in the development of wooden breast and white striping, confirming a hypoxic tissue environment in affected breast muscle (Malila et al., 2019).

Hypoxia and its sensing in the body

Hypoxia is a condition in which oxygen supply is not sufficient to maintain physiological homeostasis. The oxygen (O₂) homeostasis system in the body is important in that it is involved in energy production and metabolic signaling. Oxygen tension in the body is defined by the pO₂ that is dissolved in serum. The oxygen tension in skeletal muscle is approximately 46 mmHg, which is significantly lower than atmospheric oxygen level (160 mmHg) (Ast et al., 2019). The skeletal muscle oxygen tension can drop to as low as 3-23 mmHg by temporal fluctuations, intense exercise, disease, and high altitude, indicating that skeletal muscle tissue has the capacity to adapt to low oxygen tension and can maintain some level of function and energy production even under hypoxic conditions (Richardson et al. 2001; Ast et al., 2019). HIF protein is stabilized following hypoxia when the oxygen tension drops below the physiological level, oxygen availability is limited, and/or metabolic oxygen consumption exceeds oxygen capacity. HIF has two major subunits, HIF-1 α (oxygen-sensitive) and HIF-1 β (oxygen-insensitive) (Jaakkola et al., 2001; Semenza, 2003). Under normoxia, HIF-1 α is continuously degraded by the ubiquitin-proteasome pathway even though it is expressed (Ivan et al. 2001). HIF-1 α is degraded due to two processes. Firstly, hydroxylation of proline residues of HIF-1 α by oxygen-dependent prolyl-4-hydroxylases (PHD). Then, E3 ubiquitin ligase binds to the hydroxylated HIF-1 α and elicits proteasomal degradation. Under hypoxia, however, HIF-1 α is constantly synthesized. Inhibited PHD activity allows to translocate the HIF-1 α to the nucleus, where it forms a heterodimer with HIF-1 β . The heterodimeric complex is localized to hypoxia response elements, resulting in transcriptional upregulation of target genes (Lee et al., 2019).

The role of HIF-1 α in the response to muscle injury

Under hypoxia, the stabilization of HIF-1 α protein in skeletal muscle promotes capillarization by inducing vascular endothelial growth factors (VEGF) expression (Ameln et al. 2005). VEGF, a target gene of HIF-1 α , plays a pro-angiogenic role (Messina et al.

2007). During muscle regeneration, the expression of HIF-1 α and VEGF is increased during muscle regeneration to restore vascularization impaired by cardiotoxin and barium chloride injection (Hotta et al. 2018; Drouin et al. 2019). Intriguingly, HIF-1 α and VEGF are reported to contribute to fibrosis in damaged skeletal muscle. Muscle fibrosis can be induced when muscle regeneration is delayed under hypoxia. This is supported by that HIF-1 α induces profibrotic cytokine expression by crosstalk with TGF- β (Valle-Tenney et al., 2020). In addition, when DMD-derived fibroblasts were treated with exogenous VEGF, myofibroblasts markers (alpha-smooth muscle actin, type I collagen, connective tissue growth factor, and fibronectin) were increased (Gutpell and Hoffman. 2015). Furthermore, hypoxia-induced HIF-1 α induces the proliferation and differentiation of SCs.

Impact of hypoxia on satellite cells

In the stem cell microenvironment, a hypoxic niche is an important factor enabling various stem cells to maintain stemness and regulate self-renewal, proliferation, and differentiation (Hubbi et al., 2015). Similarly, several studies have shown that hypoxia regulates the proliferation and differentiation of SCs in mice, rats, and bovines (Kook et al., 2008; Urbani et al., 2012). Chakravarthy et al. (2001) found that 3% O₂ significantly increased the proliferation rate of primary SCs and induced the formation of larger myotubes than 21% O₂. Additionally, they reported that central regulators of cell proliferation, such as activated Akt, G1/S cyclins, and cyclin-dependent kinase, were upregulated, whereas p27, known as a cell cycle inhibitor, was downregulated under hypoxic conditions. Yun et al. (2005) confirmed that myogenesis is capable of adaptations to hypoxia. They induced myotube formation of C2C12 cells under hypoxia conditions (0.01%, 0.5%, or 2% O₂) and then transferred myotube culture to a 21% O₂ chamber. As a result, myogenic potential was strongly suppressed at all low oxygen levels compared to 21% O₂, and 21% O₂ culture after hypoxia rescued the myogenic potential. Moreover, the myogenic program of differentiating myoblast cultured at 0.5% O₂ for 12 days was initially inhibited, but extensive myogenic differentiation occurred from 6 days to 12 days. The suppression of MyoD and myogenin under hypoxia is a transient effect that can be rescued in an oxygen-dependent manner, which demonstrated that myoblast could adapt to chronic hypoxia. Data reported by Di Carlo et al. (2004), similar to Yun's results, indicated that myoblasts can recover their proliferation and myogenic potential when normal oxygen levels are restored even after the myogenic program is inhibited by hypoxia. However, Yun insisted that hypoxia represses MyoD mRNA by inhibiting the deacetylation of histones involved in MyoD promoter, whereas Di

Carlo argued that hypoxia decreased MyoD expression by increasing MyoD protein degradation rate.

HIF directly regulates the behavior of SCs through signaling pathways and epigenetic regulation. HIF-1 α stabilization promotes the maintenance of muscle stem cell populations by directly binding to the Notch intracellular signaling domain (Gustafsson et al., 2005). HIF-1 α deletion in SCs results in enhanced myogenesis and promotes muscle regeneration (Majmundar et al. 2015), whereas HIF-1 α and HIF-2 α double knockout in SCs result in delayed muscle regeneration. Interestingly, transient inhibition of HIF-2 α enhanced muscle regeneration through increased myoblast proliferation, whereas long-term inhibition produced the opposite result (Xie et al., 2018). Additionally, hypoxia induces SCs to produce myostatin, a transforming growth factor- β superfamily member. Myostatin inhibits proliferation and promotes myogenic differentiation through decreasing MyoD expression (Hayot et al., 2011; Hauerslev et al., 2014). In the case of epigenetic regulation, hypoxia activates histone deacetylase that regulates autophagy-related genes. This directly regulates the canonical Wnt/ β -catenin signaling, leading to impaired myogenesis. However, the impaired myogenesis was restored by inhibition of histone deacetylase (Zhang et al., 2019). Additionally, hypoxia induces H3K27 methylation by inactivating lysine demethylase 6A, thereby suppressing the transcriptional activation of myogenin (Chakraborty et al., 2019).

During chicken embryonic development, hypoxia can also act as a potent inhibitor of myogenic differentiation by inhibiting myogenic regulatory factors. Effects of hypoxia on the muscle maturation process vary depending on timing and oxygen concentration (Beaudry et al., 2016; Halevy, 2020). Although hypoxia challenges may be overcome before hatch, post-hatch muscle regeneration mechanisms can be affected in muscles damaged by hypoxia (Oviedo-Rondón et al., 2020). A recent paper demonstrated that hypoxia partially exacerbates wooden breast by switching satellite cell fate from a myogenic to an adipogenic program (Emami et al., 2021). In the study, proteins involved in fatty acid metabolism, such as ACYL, FASN, and ME, are significantly increased in SCs exposed to hypoxia. In addition, broilers raised at high altitudes have significantly higher incidence and severity of wooden breast with higher body weight and breast muscle yield than controls. This data may partially explain the progression of wooden breast and ectopic lipid infiltration related to hypoxia.

Impact of hypoxia on fibroadipogenic progenitors

Several studies have examined the effects of hypoxia on skeletal muscle SCs, but only a few have investigated the proliferation and differentiation of FAPs. Fibroadipogenic progenitors have multipotency, being able to differentiate into adipocytes and fibroblasts. Given the localized hypoxic environment characteristics of broiler myopathies, hypoxia may affect aberrant differentiation of FAPs by regulating transcription factors involved in adipogenesis or fibrogenesis, resulting in fibrofatty tissue accumulation. Hypoxia significantly increases the number of FAPs colony-forming units, indicating that FAPs have a higher proliferative capacity under hypoxia (Drouin et al., 2019). In addition, FAPs isolated from injured muscle in a hypoxic state display suppressed adipogenic potential and enhanced osteogenic potential (Drouin et al., 2019; Ollitrault et al., 2021). This suggests that hypoxic conditions can contribute to changes in FAPs phenotype. However, despite the importance of FAPs in muscle growth and regeneration, studies on proliferation and fate decisions regulated by hypoxia have been conducted to a very limited extent. Moreover, studies related to the broiler myopathies have not yet been conducted. Therefore, it is imperative to understand the proliferation and differentiation of broiler FAPs in hypoxic conditions.

Similarities between myopathies in human and broiler

Chickens have been widely used as a viable model for studies of developmental biology and human diseases, including obesity and type 2 diabetes. Commercial broilers are characterized by excessive deposition of abdominal fat and rapid growth of breast muscle due to strong genetic selection for growth. However, broiler myopathies, such as white striping and wooden breast, are associated with localized tissue hypoxia characterized by a higher $p\text{CO}_2$, lower $p\text{O}_2$, and lower $s\text{O}_2$ (Lake et al., 2020).

Similarly, respiratory failure is a common feature in many human myopathies. Respiratory failure is defined as the inability of the respiratory system to perform its basic functions properly (i.e., oxygenation failure and carbon dioxide elimination). In muscular dystrophies, respiratory failure is often the leading cause of death alongside heart problems, due to the inability of the respiratory system to function properly (Nguyen et al., 2021). The incidence and severity of respiratory disorders vary according to the type and progression of muscular dystrophies. Facioscapulohumeral muscular dystrophy and Emery–Dreifuss muscular dystrophy have a very close relationship with respiratory disorders. Additionally, DMD patients and muscular dystrophy X-linked mice display reduced capillary density, and

the mouse SCs have lower angiogenic capacity and reduced VEGF and HIF-1 α expression (Buckley et al., 2013; Rhoads et al., 2013).

Duchenne muscular dystrophy and Facioscapulohumeral muscular dystrophy are characterized by hypoxia-induced mitochondrial dysfunction and alterations in mitochondrial ultrastructure. This leads to impaired energy metabolism, oxidative stress, and cell apoptosis (Turki et al., 2012; Dubinin et al., 2020). Similarly, affected broiler breast muscle exhibits damaged mitochondria and increased fatty acids synthesis. As a result, this induces increased myofiber degeneration, fiber hypertrophy, lipidosis, and fibrosis (Ouali et al., 2013; Kozakowska et al., 2015; Hosotani et al., 2020). These pathological features that characterize breast myopathies are also found in myopathies of various origins in humans, such as Duchenne muscular dystrophy and age-related sarcopenia. While ectopic lipid accumulation in skeletal muscle is associated with type 2 diabetes in humans, this feature is not a comparable metabolic risk factor for broiler myopathies. This is because lipotoxicity caused by this phenotype leads to muscle atrophy in humans, which is inconsistent with muscle hypertrophy in chickens with myopathy (Lake et al., 2020).

According to Hosotani et al. (2020), severe fibrotic areas and myofibers of affected broiler breast muscle exhibit rimmed vacuoles and impaired autophagy/mitophagy. These autophagic vacuoles are also observed in lysosomal glycogen storage myopathies, including Pompe and Danon diseases, which are mitochondrial disorders. The mutations or deletions in mitochondrial DNA can cause mitochondrial myopathies, as muscle cells particularly require high energy. Recent studies suggest that hypoxia negatively affects mitochondrial DNA, leading to impaired mitochondrial respiration, increased mitophagy, and cell apoptosis (Wu et al., 2015; Otandault et al., 2020). Impaired mitophagy is observed in muscular dystrophy X-linked mice, resulting in the accumulation of damaged mitochondria in muscle cells and decreased expression of mitophagy-related genes (Kuno et al., 2018; Sebori et al., 2018). Mitophagy is an adaptive metabolic response that reduces the production of reactive oxygen species in order to prevent cell death. However, chronic hypoxia conditions, such as those observed in wooden breast or specific myopathies, can weaken HIF-1 α signaling and reduce mitochondrial clearance. This leads to the accumulation of damaged mitochondria, which can aggravate the muscle microenvironment and result in the occurrence of rimmed vacuoles in myofibers (Hosotani et al., 2020).

In recent years, many genomic studies using broiler breast muscle have been conducted to understand the molecular mechanisms of myopathy development. In addition to histological comparison, I listed whether the genes and proteins that were differentially

expressed in broiler myopathies have important roles in human myopathies (Table 1). This will help us to understand the molecular mechanisms of broiler myopathy by using the information derived from human myopathy studies.

Appendix 1

Table 1. List of genes and proteins associated with wooden breast and human skeletal muscle myopathies

Name	Function	Human Myopathies	Features	Reference
<i>DNM2</i>	- Endocytosis Cell motility - Cytokinesis - Apoptosis - Stimulation of GTPase	- Centronuclear myopathy - Charcot-Marie-Tooth neuropathy	- Fiber hypertrophy - Endomysial fibrosis - Mitochondrial enlargement - Mitochondrial dysfunction - Internalization of myonuclei	Lake (2021) Velleman (2022)
<i>GOT1</i>	- Replenishment of aspartate - Regulation of concentrations of glutamate - Production of non-collagenous glycoprotein (laminins)	Sarcopenia	Loss of muscle mass, quality, and strength	Lake et al. (2021) Kuttappan (2017) Kong (2021)
<i>LAMA2</i> <i>LAMA4</i>	- Regulation of the fate decision of mesenchymal stem cells - Myoblast proliferation and differentiation	Congenital muscular dystrophies	- Atrophy - Muscle weakness - Lack of muscle tone (hypotonia) - Little spontaneous movement Joint deformities (contractures)	Bordini (2021)
<i>FBN1</i>	maintenance of the functionality and stability of connective tissue	Marfan syndrome	- Weakened connective tissue - Muscle fatigue	Bordini (2021) Praud (2020) Soglia (2022)
<i>COL4A1</i> <i>COL4A2</i>	Collagen type IV heterotrimers production	COL4A1/COL4A2 syndrome	- Endomysial fibrosis - Infiltration of inflammatory cells - Fiber splitting - Internalization of myonuclei	Bordini (2021)
<i>Desmin</i>	Maintenance of cell integrity	Desminopathies	- Lipidosis - Fibrosis - Inability of muscles to regenerate	Xue Zhang (2021)
<i>CCDC85A</i>	Degradation of β-catenin	Spinal muscular atrophy	Excessive extracellular matrix accumulation	Phillips (2021)
<i>TGFB3</i>	- Cell proliferation - Cell differentiation - Apoptosis - Extracellular matrix formation - embryogenesis	- Duchenne muscular dystrophy - Amyotrophic lateral sclerosis	- Increased collagen production - Fibrosis	Phillips (2021)
<i>CAPN3</i> <i>PPP1R3A</i>	Intracellular protease	Limb-girdle muscular dystrophy type 2A	Muscle weakness	Praud (2020)
<i>SGCB</i>	Formation of sarcoglycan protein complex	- Duchenne muscular dystrophy - Limb-girdle muscular dystrophy	Muscle weakness	Pampouille (2018)

Abbreviations: DNM2, dynamin-2; GOT1, glutamic-oxaloacetic transaminase 1; LAMA2, laminin subunit alpha-2; LAMA4, laminin subunit alpha-4; FBN1, fibrillin 1; COL4A1, collagen alpha-1; COL4A2, collagen alpha-2; CCDC85A, coiled-coil domain containing 85A; TGFB3, transforming growth factor beta-3; CAPN3, calpain-3; PPP1R3A, protein phosphatase 1 regulatory subunit 3A; SGCB, sarcoglycan beta.

**CHAPTER I: AN OPTIMIZED METHOD TO ISOLATE, PURIFY,
AND DIFFERENTIATE SATELLITE CELLS FROM BROILER
CHICKS**

This manuscript has been published in similar form with co-authors Minjeong Kim, Elizabeth Shepherd, and Brynn H. Voy. in the Journal of Animal Science 100(12), 2022 (<https://doi.org/10.1093/jas/skac342>).

Abstract

Development and maintenance of healthy muscle fibers rely on the myogenic potential of SCs, muscle stem cells that proliferate and differentiate to form myotubes. Satellite cells are indispensable for post-hatch muscle growth as well as muscle repair and regeneration when myofibers are damaged. Pectoralis major of young broiler chicks (5 day-olds) is a readily available source of SCs, which can be used in vitro to elucidate cellular and molecular mechanisms responsible for muscle growth and regeneration in broilers. Here, we optimized a method for efficient isolation, purification, and differentiation of SCs, from young broiler chicks. This procedure includes a simple method that allows SCs to be purified from other muscle cell types that can impede the fidelity of follow-on experiments, particularly highly sensitive measures such as RNAseq. The methods for culturing and differentiating SCs into multinucleated myotubes were also optimized by testing serum types, concentrations, and the effects of chicken embryo extract. Using the isolation procedure, a highly pure SCs population ($94.6 \pm 2.11\%$ Pax7⁺) with high viability and yield was obtained, and their capacity to differentiate into myotubes was confirmed. Enrichment for SCs and myogenic capacity were maintained through multiple passages and after cryopreservation. Analysis of gene expression over the first 48 h of differentiation confirmed that SCs exhibited the expected molecular signature of myogenesis. Taken together, this method simplifies the ability to isolate and maintain a relatively pure population of SCs with strong myogenic potential from young broiler chicks, and should support downstream applications for assessing the impact of nutrients, metabolites, and other physiological cues on muscle growth and development in broilers.

Introduction

Muscle growth and repair rely on the behavior of SCs (also known as adult myoblasts), myogenic stem cells that reside between the plasma and basement membranes of muscle fibers (Mauro, 1961). Myogenesis can be reproduced in SCs in vitro, which makes culture of SCs a valuable tool for identifying factors that affect muscle development. Satellite cells are particularly relevant for studies in broiler chickens due to the prevalence of wooden breast and other myopathies that are thought to arise from effects of an aberrant

tissue microenvironment on SCs behavior (Caldas-Cueva and Owens, 2020; Natarajan et al., 2010). Methods for isolation of SCs begin with enzymatic digestion of muscle tissue and yield not only SCs, but also fibroblasts, immune cells, and endothelial cells. This heterogeneity can compromise downstream experimental applications, particularly those that use highly sensitive molecular assays. The lack of validated commercial antibodies against SCs surface proteins for chicken and most livestock prevents the use of cell-sorting techniques that are commonly used to purify SCs in humans and rodents. As an alternative, we developed a method to purify SCs based on differential adherence to tissue culture plastic, following a method originally developed for use in rodents (Richler and Yaffe, 1970). We also optimized the enzymatic digestion process, which varies widely across published protocols for use with pectoralis major tissue from very young chicks, to capture cells during an active period of proliferation and myogenic differentiation. This is important because the composition of connective tissue varies with age and muscle type, which impacts the type and concentrations of enzymes that are optimal for tissue digestion (Gharaibeh et al., 2008). We have also optimized the media composition to support proliferation and differentiation, and we demonstrate that these cells readily undergo myogenesis and form large, multinucleated myotubes in vitro. Finally, we have used targeted RNA sequencing to assess the sequential changes in expression of key myogenic genes across the early stages of differentiation.

Materials and methods

Isolation of satellite cells

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee-Knoxville (protocol no. 2164). Cornish Cross chicks (4-5 d) were euthanized by CO₂ asphyxiation. The skin over the breast was wiped with chlorhexidine and 70% ethanol before dissection of breast muscle. In a cell culture hood, approximately breast muscle was removed and rinsed twice in ice-cold 20% Povidone in Hank's balanced salt solution (HBSS; Gibco, Waltham, MA), cleaned of any non-muscle tissue, cut into 1 to 2 mm³ pieces, and then transferred into ice-cold HBSS containing 100 IU/mL penicillin/streptomycin (P/S; Gibco, Waltham, MA) and 2.5 µg/mL amphotericin B (AmpB; Thermo Fisher Scientific, Waltham, MA). All subsequent solutions used for isolation contained P/S and AmpB. Minced muscle tissues were centrifuged at 400 g for 5 min and digested in HBSS containing 250 CDU/mL collagenase II (Sigma, St. Louis,

MO), 2.5 mM CaCl₂, 100 µg/mL DNase I (Roche, Indianapolis, IN) at 37.5°C for 40 min to partially dissociate muscle tissue. The digest was then filtered through a 0.2 µm cellulose acetate filter (Thermo Fisher Scientific, Waltham, MA), and the partially digested tissue was gently passed through a 1-mL pipette tip with the end cut off to promote tissue dissociation. Dissociated muscle tissue was collected by centrifugation at 300 g for 5 min and subjected to a second enzymatic digestion step in HBSS containing 0.27 U/mg collagenase D (Roche, Indianapolis, IN), 0.5 U/mg Dispase II (Sigma, St. Louis, MO), 2.5 mM CaCl₂, and 100 µg/mL DNase I with shaking on an orbital shaker (160 to 240 rpm) at 38°C for 50 min. In this step, Collagenase D and Dispase II are used to preserve the integrity of surface proteins and cell membranes (Danoviz and Yablonka-Reuveni, 2012). Digestion was stopped by adding an equal volume of DMEM/F12 containing 10% fetal bovine serum (FBS; Gibco, Waltham, MA), and the digest was centrifuged at 55 g for 2 min to collect cell suspension. Then, the digested muscle was resuspended in ice-cold HBSS and centrifuged to collect additional cell suspension. The pooled suspension was dissociated into single cells by aspirating through an 18-gauge needle and passing through 70-µm and then 40-µm cell strainers (SPL Life Sciences, Pocheon, Korea). Cells were pelleted by centrifugation and red blood cells lysed following the manufacturer's protocol (Roche, Indianapolis, IN). After lysis, pellets were resuspended in DMEM/F12 containing 10% FBS.

Purification by preplating and cryopreservation

Purification by preplating was accomplished by plating isolated cells on standard tissue culture plastic and allowing cells to adhere for 40 min, following a method described for SCs isolation from rats (Richler and Yaffe, 1970). The supernatant was transferred into a collagen I-coated dish (Corning, Tewksbury, MA) and the dish was incubated for 50 min at 38°C in a CO₂ incubator with intermittent swirling to prevent promiscuous adhesion. Media containing unattached cells was then collected and centrifuged at 880 g for 5 min. If to be used for subsequent culture, cell pellets were resuspended in growth media (GM) containing Ham's F10, 20% FBS, 10% non-heat activated horse serum (HS, Gibco, Waltham, MA), 1% chicken embryo extract (CEE), 2.5 ng/mL basic fibroblast growth factor (Stemcell Technologies, Vancouver, British Columbia, Canada), and 5 µg/mL gentamicin (Lonza, Basel, Switzerland). Chicken embryo extract was prepared from 10-day-old whole embryos following a previously described method (Yablonka-Reuveni, 1995), aliquoted and frozen at -80 °C until use. Prior to use, it was centrifuged at 17,000 g for 30 min at 4 °C and passed through a 0.2 µm cellulose acetate filter (Thermo Fisher Scientific, Waltham, MA).

For cryopreservation, pellets were resuspended in 90% HS (Thermo Fisher Scientific, Waltham, MA) plus 10% dimethyl sulfoxide (Sigma, St. Louis, MO) at a density of $\sim 2 \times 10^6$ cells/mL, transferred to cryotubes, and stored in liquid nitrogen for future use.

Culture and passage

Cells were seeded at 3×10^5 on 100 mm collagen I (Corning, Tewksbury, MA)-coated dishes and allowed to attach for two days before replacing GM. Afterwards, GM was replaced every day. When cells reached 60% confluence, they were trypsinized with 0.1% trypsin and split 1:2 or 1:3 for passaging.

Immunocytochemistry

Purity of the SCs population was assessed by immunostaining with Pax-7 (AB_528428, Developmental Studies Hybridoma Bank, Iowa City, IA), a marker for SCs (Kawakami et al., 1997). Cells were seeded in a collagen-coated 96-well plates and allowed to attach, then fixed in 4% paraformaldehyde for 10 min. Fixed cells were permeabilized in 0.1% Triton X-100 (Sigma, St. Louis, MO) for 10 min at room temperature (RT), blocked in 2% BSA (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) for 30 min at RT, and then incubated with primary anti-Pax7 (1:5) at 4°C overnight in the dark. Immunostained Pax7-positive cells were visualized using a secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA), and nuclei were stained using Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA). Stained cells were photographed with an EVOS cell imaging system (Thermo Fisher Scientific, Waltham, MA). Quantification of Pax7-positive nuclei relative to the total number of nuclei was determined with ImageJ (imagej.nih.gov).

Myogenic differentiation and HEMA3 staining

Satellite cells (2×10^5 cells) were seeded on collagen I-coated 12-well plates and cultured until 70% confluency was reached. Cells were then washed in PBS and myogenic differentiation was induced by replacing GM with differentiation media (DMEM/F12 containing 10% HS and 2% CEE). Differentiation media was changed every day. Myotube formation was assessed by visually staining cells with HEMA3 (Thermo Fisher Scientific, Waltham, MA), a shelf-stable and ready to use stain that was shown to robustly stain myotubes in primates (Levitt et al., 2019). Cells were rinsed twice with PBS, fixed in methanol at RT for 10 min, and stained with HEMA3 according to the manufacturer's

directions. Multinucleated myotubes were visualized under an EVOS microscope (Thermo Fisher Scientific, Waltham, MA).

Targeted RNA sequencing

Cells were harvested immediately prior to induction of differentiation and at 12 h, 24 h, 36 h, and 48 h after differentiation induction for isolation of RNA. Total RNA was isolated using Direct-zol RNA Miniprep kit according to the manufacturer's instructions (Zymo Research, Irvine, California) and quality was verified by visualization using gel electrophoresis. Targeted RNA sequencing was used to profile expression of 14 genes in myogenesis using methods we have previously described (Mihelic et al., 2020). Reads were normalized to the geometric mean of four housekeeping genes (Actb, Tbp, Gapdh, and Ywhaz) and log transformed prior to statistical analyses.

Statistical Analysis

Statistical analyses for proliferation assay and immunocytochemistry were performed using PASW Statistics 18 Software (SPSS Inc., Chicago, IL). Targeted RNA sequencing data were analyzed using routines in R (version 4.1.3). Data were analyzed by one-way ANOVA with Tukey's test to assess the significant pairwise differences between treatments. *P*-values less than 0.05 were considered to be statistically significant.

Result and Discussion

Cells isolated using this protocol exhibited the expected round, small, and refractive morphology after adhering to collagen-coated dishes (Fig. 1, left). After attachment to the surface of collagen-coated dishes, cells adopted a slightly elongated spindle-shape as expected for SCs (Fig. 1A, left). In contrast, cells that rapidly adhered to tissue culture plastic were flat and larger, with morphology consistent with fibroblast-like cells (Fig. 1A, right).

Validation of pre-plating to enrich for satellite cells

Cells isolated using this procedure, which typically yields $6 - 7 \times 10^6$ cells / 400 mg of tissue, were evaluated for expression of the hallmark gene, Pax7, a transcription factor that maintains SCs in their undifferentiated state (Fig. 1B) (Zammit et al., 2006). In the initial population of cells, before preplating, approximately 72% of cells were Pax7⁺ (Fig. 1C). The first preplating step (on tissue culture plastic) significantly increased this percentage to ~79% (*P* < 0.05), and the second preplating step (on collagen) further increased purity, with 91% of cells positive for Pax7. Pax7 immunostaining was also used to confirm cells

maintained their SCs features after passaging. As shown in Fig. 1C, using pre-plating after trypsinization was sufficient to maintain and improve SCs enrichment (up to ~ 94.6%) in passaged cells. It also should be noted that we used F10-based media during routine growth and SCs expansion because it has been reported to give myoblasts a growth advantage over fibroblasts (Rando and Blau, 1994), likely due to a lower concentration of calcium that suppresses differentiation of SCs (Shainberg et al., 1969). In our experience, cells isolated using this method can be passaged at least three times, maintaining their ability to rapidly proliferate and to undergo myogenic differentiation with each passage. Interestingly, the time required for SCs to attach to collagen-coated dishes and to begin to proliferate seems to decrease as passage number increases.

Myogenic differentiation

Various types and amounts of serum have been used to differentiate SCs from young chicks. Therefore, effects of different sources (HS or FBS) and concentrations (2%, 5%, or 10%) of serum, with or without CEE (2%) were evaluated by staining myotubes with HEMA3 (Fig. 2). Cells proliferated equally well in HS and FBS. As a rich source of growth factors, inclusion of 2% CEE promoted SCs proliferation regardless of serum type (data not shown). Increasing serum concentration enhanced both the intensity of staining and size of myotubes. Horse serum induced cell fusion and differentiation more effectively than FBS at all serum concentrations, based on the density of staining and size of myotubes. Inclusion of CEE (2%) further enhanced density and size, irrespective of serum source. Commercially available sources of serum and CEE are relatively expensive, while preparation of CEE in house is inexpensive but relatively labor intensive. Therefore, 1-2% of CEE can be used to augment proliferation and differentiation while still minimizing use.

DEGs involved in myogenesis

Changes in gene expression across the 48 h of myogenesis reflected the rapid induction of differentiation. Eleven of the 14 genes profiled were differentially expressed across time points. Genes considered to represent the molecular signature of both quiescent (*Myf5*, *Pax3*, and *Pax7*) and activated (*Myod1*) SCs were downregulated across myogenic differentiation (Fig. 3). In parallel, *Myh1b* and *Myh1d*, both of which are induced during myotube formation, were upregulated throughout differentiation. Finally, *Myh1e*, *Myog*, and *Mef2c* were upregulated 12 h after differentiation induction, and then returned to baseline

levels. These changes in gene expression further confirm that SCs isolated using this procedure faithfully reproduce the process of myogenesis.

Conclusion

We established a method that allows efficient isolation of a relatively purified population of SCs from breast muscle of young broiler chicks. Cells isolated by this method exhibit a high potential for myogenic differentiation and readily form multinucleated muscle fibers in vitro. While culture and differentiation of SCs in vitro cannot replicate the complex multicellular interactions that occur in vivo, in the context of muscle tissue, this model is valuable for the ability to focus specifically on myogenesis and how the process is impacted by exogenous conditions, nutrients, and other factors. In light of myopathies in broilers, SCs are informative for understanding how specific factors such as hypoxia impact the satellite cell processes that are required for muscle repair. This enhanced isolation and purification technique will therefore aid in further dissecting the molecular and metabolic mechanisms that alter myogenesis in broiler breast muscle, and are potentially involved in the etiology of myopathies (e.g., white striping and wooden breast).

Appendix 2

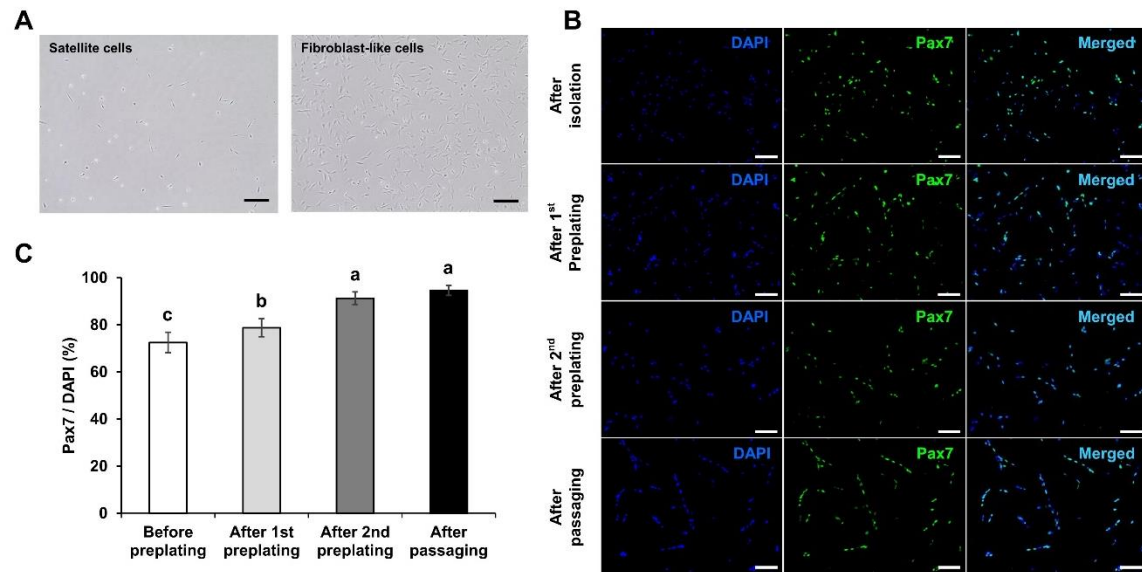


Figure 1. Conformation of purity of broiler satellite cells isolated from pectoralis major tissue

Phase contrast microscopic images showing morphology of (A, left) satellite cells on a collagen I-coated dish and (A, right) fibroblast-like cells on a tissue culture plastic. Scale bar = 200 μ m. (B) Representative images of immunostained Pax7-positive cells. Cells were stained with Pax7 (green), DAPI (blue), and merged (light blue). Images were taken after each purification step. Scale bar = 100 μ m. (C) Quantification of the cells stained for Pax7. The number of nuclei was counted by ImageJ. Error bars = SD. $n = 10$. $P < 0.05$.

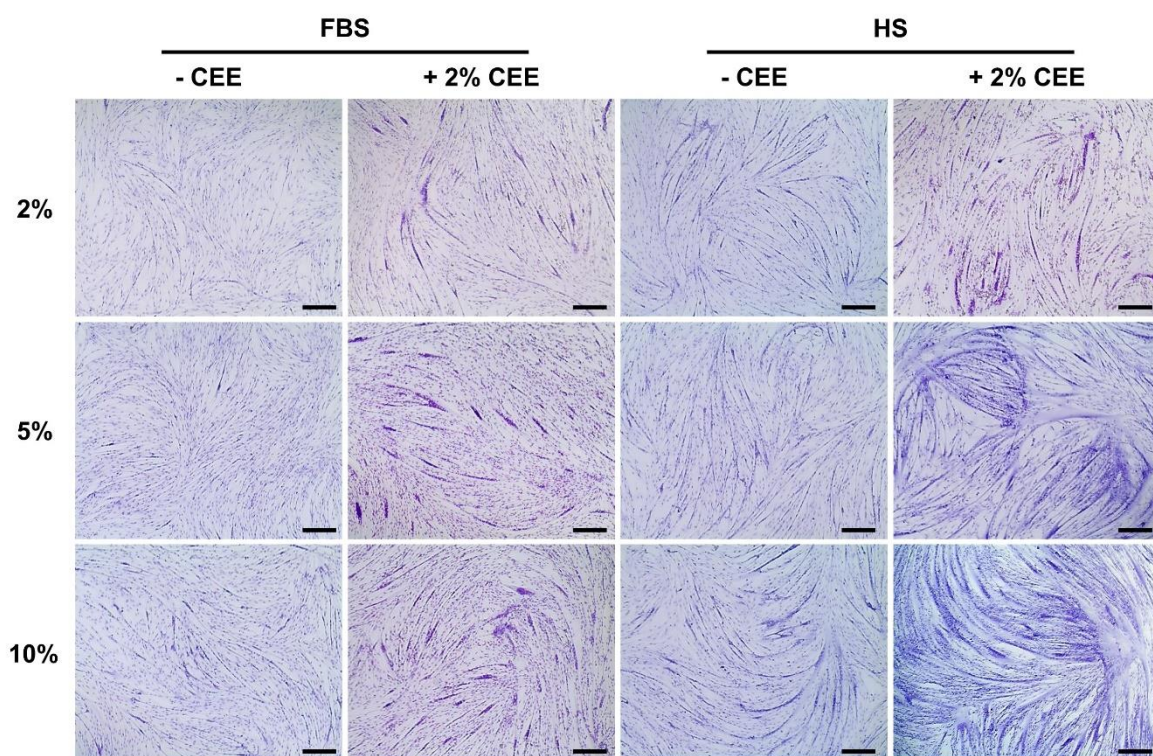


Figure 2. Myogenic differentiation of broiler satellite cells

Differentiation was induced in media containing different serum types and concentrations for 3 days. Fused myonuclei were stained with HEMA3. Scale bar = 500 μ m.

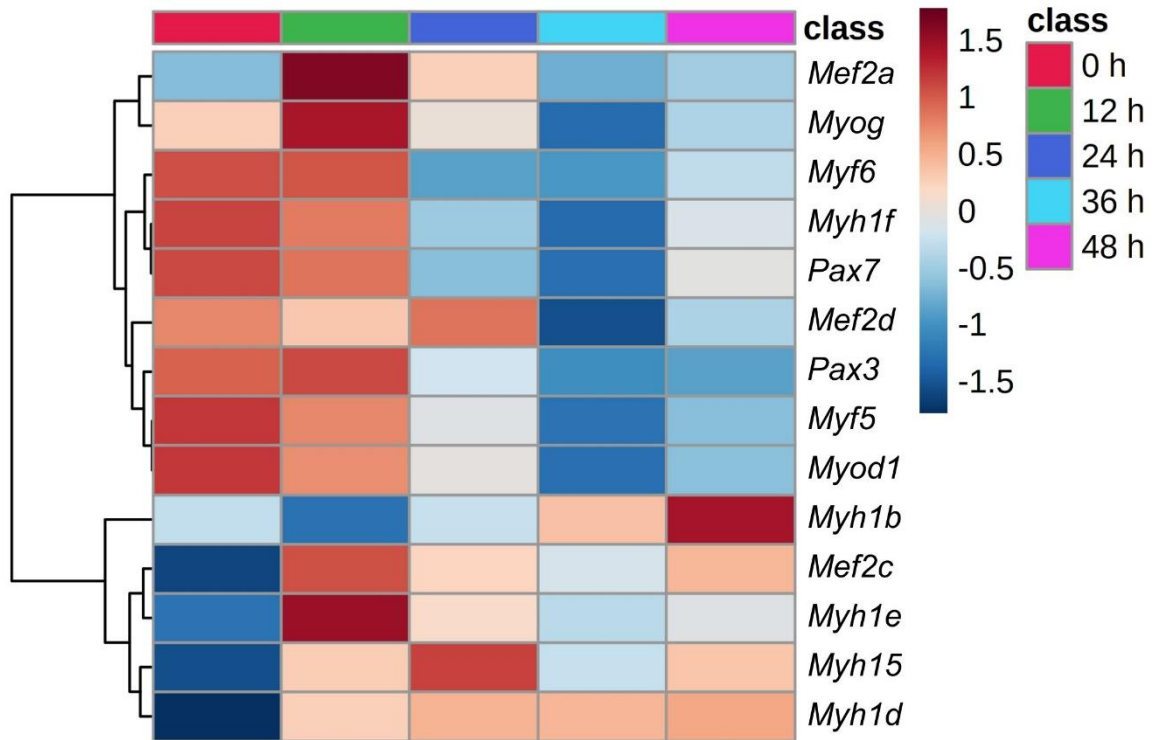


Figure 3. Time-series clustering of differentially expressed genes in differentiating broiler satellite cells

Satellite cells were harvested at 0 h, 12 h, 24 h, 36 h, and 48 h after myogenic differentiation induction for RNA isolation. $n = 6$ per time point. The color indicates the relative expression value.

**CHAPTER II: ISOLATION AND CHARACTERIZATION OF
FIBROADIPOGENIC PROGENITORS FROM PECTORALIS MAJOR
OF BROILER CHICKS**

This manuscript is being prepared for publication with co-authors Minjeong Kim, Lindsay Brown, Shawn Campagna, and Brynn H. Voy. for Plus One.

Abstract

Fibroblast adipogenic progenitors, PDGFR⁺ interstitial muscle-resident mesenchymal stem cells, play a crucial role in maintaining muscle homeostasis and supporting healthy muscle repair. Cues within the muscle environment can induce aberrant differentiation of FAPs into adipocytes, which is thought to disrupt the muscle repair process and contribute to the development and pathogenesis of broiler myopathies characterized by fibrofatty infiltration. However, the FAPs population has not yet been characterized in chickens. This study aims to optimize a simple and efficient method to isolate FAPs from the pectoralis major of young broiler chicks and to characterize adipogenic differentiation of FAPs by comparing morphologically and biochemically with subcutaneous preadipocytes (PAs). We tested supplemental effects of oleic and linoleic acids, differentiation cocktails (3-isobutyl-1-methyl-xanthine, dexamethasone, and insulin), or rosiglitazone on induction of adipogenic differentiation of both cell types. As a result, total lipid accumulation in FAPs was regulated by the same supplements that controlled lipid accumulation in PAs. However, these supplements did not affect the lipid accumulation per cell; instead, they resulted in an increased average lipid droplet size in both FAPs and PAs. Notably, FAPs exhibited lower lipid content per cell and smaller average lipid droplet size compared to PAs. Furthermore, we observed that differentiated FAPs had higher levels of eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C20:6n-3). However, there were no significant differences in the percentages of total saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids between FAPs and PAs. By employing targeted RNA sequencing analysis, we were able to identify and compare the adipogenic programs of both cell types, revealing both similarities and differences. Altogether, this study provides an isolation method for broiler FAPs and fundamental knowledge of the adipogenic characteristics of broiler FAPs. Thus, findings from this study may provide important insights into FAPs as a promising therapeutic target for broiler myopathies.

Introduction

In the last 50 years, genetic selection of commercial broilers has focused on high feed efficiency, larger breast muscle yield, and accelerated growth rate (Tijare et al., 2015). Despite these benefits for broiler production, rapid improvements have come at a cost to

quality of the breast muscle. Wooden breast and white striping are the two most prevalent forms of myopathies in common commercial lines of broilers (Shivo et al., 2014). Wooden breast lesions result when areas of the breast muscle (pectoralis major) become hardened and thickened, with hemorrhagic areas on the surface of the muscle. White striping describes white fibrotic, lipid-rich striations on the surface of the pectoralis major (Papah et al., 2017; Velleman, 2019). These features of broiler mammary muscle lesions are similar to those of human myopathies, such as Duchenne muscular dystrophy and age-related sarcopenia (Grounds et al., 2014; Moratal et al., 2019).

A muscle stem cell population known as FAPs, which are interstitial cells resident in muscle tissue, has emerged as a culprit in the etiology and pathogenesis of human myopathies. Fibroadipogenic progenitors are characterized by the surface expression of platelet-derived growth factor receptor alpha (PDGFR α), a membrane-bound tyrosine kinase receptor (Joe et al., 2010; Uezumi et al., 2010; Contreras et al., 2021). Their main function is to produce ECM proteins, such as collagen, metalloproteinases, fibronectin, laminin, and proteoglycans, which provide a supportive niche for the self-renewal of SCs (Lukjanenko et al., 2016; Rayagiri et al., 2018). Fibroadipogenic progenitors exhibit properties of mesenchymal stem cells and can differentiate into adipocytes, fibroblasts, osteoblasts, and chondrocytes, although the latter two fates are not documented to occur in vivo (Wosczyzna et al., 2012; Marinkovic et al., 2019; Eisner et al., 2020; Giuliani et al., 2021). Due to their multipotency, aberrant differentiation of FAPs could contribute to excessive fibrofatty infiltration in damaged skeletal muscles (Joe et al., 2010; Uezumi et al., 2010).

Convergent evidence suggests that FAPs may be responsible for the breast myopathies that currently plague the broiler industry. However, this cell population has not yet been characterized in chickens. Reuveni et al. (1988) demonstrated that non-myogenic cells in the breast muscle possess binding sites for PDGF, and PDGFR α was identified through QTL mapping as a candidate gene for broiler myopathies (Papah et al., 2018; Pampouille et al., 2019; Praud et al., 2020). Taken together, FAPs may contribute to excessive fibrosis and lipid deposition in broiler myopathies through aberrant differentiation.

Thus, it is necessary to investigate their cellular characteristics as well as to optimize an isolation protocol for broiler FAPs in order to gain a better understanding of their potential roles in broiler breast muscle myopathies. Here, the described protocol includes media composition to support the adipogenic differentiation, as well as a comparison of adipogenic differentiation with primary preadipocytes isolated from subcutaneous fats using lipid

droplet staining, fatty acid methyl ester (FAME) analysis, and targeted RNA sequencing. The information provided can be valuable for future studies to identify mechanisms behind fibrofatty infiltration in broiler myopathies. It can also aid in developing effective interventions to prevent or treat such conditions in broilers. The optimized protocol for broiler FAPs isolation may also have broader applications in the study of muscle stem cells in other livestock species.

Materials and methods

Animal and housing

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee-Knoxville (protocol no. 2164). Day-old male chicks (Cornish Cross) were obtained from vendors (Tractor Supply Co.). Chicks were housed at Johnson Animal Research and Teaching Unit under standard husbandry conditions. Chicks were raised in metal stackable cages, fed a commercial starter diet (Tennessee Farmers Co-Op), and provided access to feed and water ad libitum. The RT during the brooding period (0-5 days of age) was maintained above 34°C, and the light:dark cycle was 23h:1h.

Isolation of fibroadipogenic progenitors and preadipocytes

The isolation protocol of primary FAPs was developed based on a previously reported method used to isolate SCs (Jung et al., 2022) (Figure 8). To obtain breast muscle tissue, a 5 days-old chick was euthanized by CO₂ asphyxiation, and the breast skin was cleaned with chlorhexidine and 70% ethanol. About 2 grams of breast muscle were collected and washed twice with cold 20% Povidone in 1X phosphate-buffered saline (PBS). Then, unwanted tissue, including pectoral fascia and subcutaneous fat, was removed in cold Hank's balanced salt solution (HBSS) containing 100 IU/mL penicillin/streptomycin (P/S; Gibco, Waltham, MA), and 2.5 µg/mL amphotericin B (Thermo Fisher Scientific, Waltham, MA). All subsequent steps were carried out on the ice, and the muscle tissues were finely minced using single-edge razor blades. Following this, the minced muscle was digested in HBSS solution containing 250 CDU/mL collagenase II (Sigma, St. Louis, MO), 2.5 mM CaCl₂, 100 µg/mL DNase I (Roche, Indianapolis, IN), 100 IU/mL P/S, and 2.5 µg/mL amphotericin B in an orbital shaker (160 rpm) at 37.5°C for 40 min. Cold HBSS was added to digested muscle tissue followed by centrifugation at 300g for 5 min to collect the digest. This digest was further incubated in HBSS containing 0.27 U/mg collagenase D (Roche,

Indianapolis, IN), 0.5 U/mg Dispase II (Sigma, St. Louis, MO), 2.5 mM CaCl₂, 100 µg/mL DNase I, 100 IU/mL P/S, and 2.5 µg/mL amphotericin B) on the orbital shaker (160 rpm) at 38°C for 50 min. To stop enzyme activity, cold DMEM/F12 media containing 10% fetal bovine serum (FBS) (Gibco, Waltham, MA) was added to the digest. The suspension was then passed through 70-µm and then 40-µm cell strainers (SPL Life Sciences, Pocheon, Korea) and centrifuged at 880 g for 5 min to collect cell pellets. After cell pellets were resuspended in DMEM/F12 containing 10% FBS, 100 IU/mL P/S, and 2.5 µg/mL amphotericin B, primary broiler FAPs were purified from myogenic cells based on the selective adhesion to the tissue culture dish, known as preplating. Cells in suspension were allowed to adhere to a tissue culture dish for 25 min with intermittent swirling to prevent myogenic cells from adhesion. Then, media containing myogenic cells was discarded and the dish was rinsed twice in 1X PBS. Adherent FAPs were cultured in DMEM/F12 containing 10% FBS, 100 IU/mL P/S at 38.0°C in a humidified incubator, with a 5% CO₂/95% O₂. For cryopreservation, the cell pellets were resuspended in DMEM/F12 media composed of 30% horse serum (Thermo Fisher Scientific, Waltham, MA) and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) at a density of $\sim 2 \times 10^6$ cells/mL, transferred to cryotubes, and stored in liquid nitrogen (-196°C) for future use. Broiler preadipocytes were isolated as previously described (Kim et al., 2022).

Adipogenic differentiation

Primary FAPs (3×10^5 cells) were seeded on 2% (w/v) gelatin-coated 100 mm² dishes. For adipogenic differentiation, cells were briefly washed in 1X PBS and cultured in DMEM/F12 containing 10% chicken serum (CS) and 100 IU/mL P/S. We tested the supplemental effects of a fatty acid mixture (94 ng/mL linoleic acid, 94 ng/mL oleic acid), adipogenic cocktail (500 µM 3-isobutyl-1-methyl-xanthine, 250 nM dexamethasone, 1 µg/ml insulin), or 2 µM rosiglitazone on adipogenic differentiation. Adipogenic differentiation was induced for 5 days. Lipid droplets were visualized by BODIPY 493/503 (Thermo Fisher Scientific) or AdipoRed (Lonza, Walkersville, MD). The number and size of lipid droplets per cell were measured with ImageJ (imagej.nih.gov). Fibrotic differentiation was induced for 3 days in DMEM/F12 supplemented with 10% FBS, 1 ng/ml TGF-beta1 (Abnova, Cambridge, UK), 50 µg/ml ascorbic acid, and 100 IU/mL P/S. The half volume of differentiation induction media was changed every day.

Immunocytochemistry and image capture

Fibroblast progenitors were assessed by immunostaining with Pax7 (1:5, DSHB, Iowa City, IA), PDGFR α (1:50, sc-398206, Santa Cruz Biotechnology, Santa Cruz, CA), CD56 (1:100, NBP2-66968, Novus Biologicals, Littleton, CO), and CD45 (1:100, MCA2413GA, Bio-Rad, Hercules, CA). Fibrogenic differentiation of FAPs was visualized by type-1 procollagen (1:50, DSHB). Cells were fixed in 4% paraformaldehyde for 10 minutes at RT. Subsequently, the fixed cells were washed in 1X PBS three times for 5 minutes each. After the washing steps, the cells were permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO) for 10 minutes at RT. Following another round of washing in PBS for 5 minutes each, blocking was performed by incubating the cells with 2% bovine serum albumin (Sigma, St. Louis, MO) in 1X PBS for 30 minutes at RT. The cells were then incubated with the primary antibody overnight at 4°C in the dark. Secondary antibodies used with a dilution of 1:200 were Alexa fluor 488 or 594-conjugated anti-goat IgG (Thermo Fisher Scientific, Waltham, MA) and PE-conjugated anti-rabbit IgG (Novus Biologicals). Nuclei were stained using Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA). Immunostained cells were photographed with an EVOS cell imaging system (Thermo Fisher Scientific, Waltham, MA).

Fatty acid profiling

Cells were induced to undergo adipogenic differentiation in DMEM/F12 containing 10% CS and 100 IU/mL P/S for 5 days. Following the induction period, the cells were flash-frozen using liquid nitrogen. Subsequently, samples were derivatized with butylated hydroxytoluene (Millipore Sigma, St. Louis, MO), an isotopically labeled internal standard (Cambridge Isotope Laboratories, Inc., Andover, MA), and boron trifluoride (14% in methanol; Millipore Sigma, St. Louis, MO). The samples were allowed to derivatize at 90°C for 90 min, after which they were cooled at RT and mixed with 1 mL of HPLC-grade water (Thermo Fisher Scientific, Waltham, MA) before being extracted with hexane. The extracted analytes were then analyzed using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA) coupled to an Exactive™ Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) using a custom interface equipped with an atmospheric pressure chemical ionization source to identify FAMES. The FAME analysis and subsequent data processing were conducted as previously reported (Powers et al., 2019). The FAMES were separated into a polyethylene glycol column (FAMEWAX, 30 m x .25 mm i.d. x 0.25 μ m df) (Restek, Bellefonte, PA) with the carrier gas, helium (flow rate at 1.7

mL/min). The data generated by the GC-MS was analyzed using the metabolomic analysis and visualization engine. Fatty acid methyl ester peaks were identified based on retention time from a standard (Supelco 37 Component FAME mix, CRM47885, Burlington, MA).

The fatty acid content in FAPs and PAs were expressed as relative percentages of the total FAMES. Enzyme activity indices for stearoyl-CoA desaturase (SCD), delta-5 desaturase (D5D), delta-6 desaturase (D6D), de novo lipogenesis (DNL), and elongation (EL) were determined by calculating product to precursor ratios. These equations included SCD, which was determined by the ratio of 16:1n-7+18:1n-9 to 16:0+18:0, D5D, which was determined by the ratio of 20:4n-6 to 20:3n-6, D6D, which was determined by the ratio of 18:3n-6 to 18:2n-6, DNL, which was determined by the ratio of 16:0 to 18:2n-6, and EL, which was determined by the ratio of 18:0 to 16:0.

Targeted RNA sequencing

Fibroblast progenitors PAs were harvested for RNA isolation at various time points, including 0 h (immediately after induction), 24 h, 48 h, 72 h, and 96 hours, following adipogenic differentiation induction. Both cells were cultured in DMEM/F12 medium supplemented with 10% CS and 100 IU/mL P/S. Total RNA was isolated using a Direct-zol RNA Miniprep kit according to the manufacturer's instructions (Zymo Research, Irvine, California). RNA concentration and quality were determined by spectrophotometry and gel electrophoresis, respectively. Total RNA (500 ng) was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Prior to library preparation for targeted RNA sequencing, cDNA quality was confirmed based on PCR for Actb (F: 5'-ATG AAG CCC AGA GCA AAA GA-3', R: 5'-GCT GTG GTG GTG AAG CTG TA-3') and visualization of products using gel electrophoresis.

Targeted RNA sequencing was used to profile expression of 685 genes involved in proliferation, adipocyte development, and metabolism using methods previously described (Mihelic et al., 2019; Jung et al., 2022). Briefly, primers were designed based on the chicken refseq genome (Gallus_gallus-7.0/galGal7) using NCBI Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) to represent the consensus transcript for each gene and to yield products that spanned exon boundaries. Reads were aligned to the reference genome and read counts were obtained using CLC Genomics Workbench (Qiagen, Germantown, MD). Then, only genes for which 80% or more of the reads in all samples were greater than the 10th percentile were filtered out. Reads were normalized to the total

counts and log transformed prior to statistical analyses in R software (Version 3.6.2.). After data processing, differentially expressed genes (DEGs) were identified using EdgeR during adipocyte differentiation of FAPs or PAs, respectively, and at all five-time points between FAPs and PAs. The general cut-off threshold of $|\log_2\text{fold change (FC)}| \geq 2$ with an FDR < 0.05 was used as the threshold for selecting upregulated DEGs during adipogenic differentiation of FAPs and PAs.

Statistical analyses

Statistical analyses were conducted using PASW Statistics 18 Software (SPSS Inc., Chicago, IL). Targeted RNA sequencing data were analyzed using routines in R (version 4.1.3). All data were presented as mean with standard deviation and analyzed using student t-test or one-way ANOVA with Tukey's test to assess significant pairwise differences between treatments. Statistical significance was determined by *P*-values less than 0.05.

Results

Cells isolated using this protocol quickly adhered to the tissue culture plastic and displayed a flattened, fibroblast-like appearance. On the other hand, myogenic cells showed poor adhesion to the tissue culture plastic and exhibited a small, round morphology. For subsequent investigations, rapidly adhering cells were expanded in DMEM/F12 supplemented with 10% FBS and 1X P/S (Figure 9A), with a doubling time of approximately 23 h.

PDGFR α ⁺ broiler fibroadipogenic progenitors efficiently differentiate into both adipocytes and collagen type-I-producing cells

After eliminating myogenic cells through preplating, broiler FAPs were confirmed based on established cell markers and multipotency. Isolated broiler FAPs were stained with CD45 as a hematopoietic marker, CD56 as an immune cell marker, Pax7 as a satellite cell marker, and PDGFR α as a muscle mesenchymal progenitor marker. The broiler FAPs were characterized by positive expression of PDGFR α and negative expression of CD45, CD56, and Pax7. PDGFR α ⁺ broiler FAPs had the potential to undergo both adipogenic and fibrogenic differentiation (Figure 5). The formation of lipid droplets in broiler FAPs increased dramatically within 24 h of adipogenic differentiation induction (Figure 5C). In addition, in the presence of TGF- β 1, broiler FAPs differentiated into collagen type-I-producing cells (Figure 5C).

Adipogenic differentiation induction in broiler fibroadipogenic progenitors and subcutaneous preadipocytes

After 5 d after adipogenic differentiation induction with CS, the lipid accumulation in FAPs was lower than that in PAs, and the average size of lipid droplets in FAPs was smaller compared to PAs (Figure 6). Supplemental fatty acids can be used to enhance the differentiation of chicken preadipocytes (Regassa et al., 2017), while cocktails of 3-isobutyl-1-methyl-xanthine, dexamethasone, and insulin is used to induce adipogenic differentiation in other species (Zebisch et al., 2012). To determine if either of these differentially enhanced lipid accumulation in FAPs vs. PAs, cells were induced to undergo differentiation for 5 d in CS supplemented with either oleic and linoleic acids, with the 3-isobutyl-1-methyl-xanthine, dexamethasone, and insulin, or with rosiglitazone. Lipid accumulation per cell (AdipoRed/NucBlue) was not affected by these supplements. However, both fatty acids and the differentiation cocktails significantly increased lipid droplet size in both FAPs and PAs (Figure 7).

Broiler fibroadipogenic progenitors and preadipocytes have distinct fatty acid profiles

Fatty acids that accumulate during adipogenic differentiation can come from de novo synthesis or from uptake. Fatty acid composition of FAPs and PAs was profiled to compare the types of fatty acids that are deposited in each cell type and their metabolism. A total of 36 FAMES were profiled in both FAPs and PAs four days after adipogenic differentiation induction with DMEM/F12 containing 10% CS and 100 IU/mL P/S (Table 2). The composition of FAMES in FAPs was similar to that in PAs, and palmitic acid (C16:0), oleic acid (C18:1), and stearic acid (C18:0), linoleic acid (C18:2n-6) were the main compositions in both FAPs and PAs (Table 2). Medium-chain fatty acids (C8 – C11) were mostly below the limits of detection in PAs but were detected in most FAPs samples. In addition, long-chain (arachidic; C20:0 and heneicosylic; C21:0) and very-long-chain saturated fatty acids (behenic; C22:0, tricosylic; C23:0, and lignoceric; C24:0) were higher in FAPs than in PAs. In terms of monounsaturated and polyunsaturated fatty acids, a lower content of myristoleic (C14:1n-5), α -linolenic (C18:3n-3), eicosatrienoic (C20:3n-3), dihomo- γ -linolenic (C20:3n-6), eicosapentaenoic (C20:5n-3), erucic (C22:1n-9), docosadienoic (C22:2n-6), docosapentanoic (C22:5n-6), and docosahexaenoic (C22:6n-3) was observed in FAPs, while the content of γ -linolenic (C18:3n-6) was higher in FAPs. Compared to PAs, the total content of n-3 polyunsaturated fatty acids was lower in FAPs, while the total content of n-6 polyunsaturated fatty acids in FAPs was not different. Despite the differences in individual

fatty acid content, no significant difference was observed in the total content of saturated, monounsaturated, and polyunsaturated fatty acids between FAPs and PAs. As shown in Table 2, FAPs showed higher D5D and D6D indices than PAs.

Transcriptome changes during adipogenic differentiation between broiler fibroadipogenic progenitors and preadipocytes

Targeted RNA sequencing was utilized to assess similarities and differences between FAPs and PAs prior to and after adipogenic differentiation. For the analysis, FAPs and PAs were induced to undergo adipogenic differentiation with DMEM/F12 containing 10% CS and 100 IU/mL P/S for 4 d. The obtained read data underwent partial least squares discriminant analysis (PLS-DA) to compare the groups (Figure 8). The PLS-DA analysis revealed a distinct separation between FAPs and PAs throughout adipogenic differentiation. A heatmap generated from 129 gene expression levels across adipogenic differentiation revealed distinct patterns of gene expression changes over time. These patterns included clusters of genes exhibiting upregulation in both cell types, downregulation in both cell types, and divergent trends between the two cell types (Figure 9). At 0 h time point, 177 genes were differentially expressed between the two cell types, including 87 genes upregulated and 90 genes downregulated in FAPs compared to PAs. Fibroadipogenic progenitors highly expressed Myogenic Differentiation 1 (Myod1; 200-fold change) and myogenin (65-fold change) than PAs. Previous studies demonstrated ectopic expression of the two genes in brown PAs, white PAs, or FAPs isolated from mice and chickens. Moreover, these two genes are considered to play inhibitory roles in adipogenesis and lipogenesis (Zhang et al., 2019; Wang et al., 2021; Biferali et al., 2021). Transcription factor 21 (TCF21), known as a white adipocyte marker, was found to be expressed 48 times more in preadipocytes compared to FAPs.

During adipogenic differentiation in PAs, expression levels of 203 of the 685 genes represented on the panel were significantly changed (FDR $p \leq 0.05$), including 98 genes upregulated and 105 genes downregulated (Figure 13). Of these upregulated PAs genes, 47 genes were selected with a fold change greater than 2. During adipogenic differentiation in FAPs, 36 genes of the 47 genes were significantly changed, including 34 genes upregulated and 2 genes downregulated, while 11 genes of the 47 genes were not significantly changed. A total of 46 genes were significantly upregulated across differentiation of FAPs, 36 genes of the 46 genes were significantly changed, including 34 genes upregulated and 2 genes downregulated, while 10 genes of the 46 genes were not significantly changed.

Discussion

Intramuscular fat plays a significant role in the health of both humans and meat-producing animals (Hausman et al., 2014). In particular, the quantity and distribution of intramuscular fat are crucial traits of meat quality, including tenderness and flavor (Hausman et al., 2009). However, excessive accumulation of intramuscular fat has been implicated as a hallmark of metabolic and inflammatory disorders in humans and broilers, such as Duchenne muscular dystrophy and wooden breast, respectively (Arrighi et al., 2015). Therefore, understanding the mechanisms behind intramuscular fat accumulation and its regulation is critical for improving both animal and human health as well as enhancing meat quality of meat-producing animals (Zhang et al., 2019; Luo et al., 2022).

Fibroadipogenic progenitors have been established to play a significant role in the formation of myopathies in humans and mice, as they are the primary cellular sources of fibrofatty tissue in skeletal muscle (Joe et al., 2010; Uezumi et al., 2010; Uezumi et al., 2014). However, to our knowledge, there has been limited characterization of FAPs in chickens, and their role in broiler myopathies has not been well investigated. Given that abnormal differentiation of FAPs could contribute to excessive fibrofatty infiltration in wooden breast-affected muscle lesions, it is crucial to conduct further research to gain a deeper understanding of the biology of broiler FAPs.

We optimized a simple and efficient isolation method for FAPs from the pectoralis major of broiler chickens and conducted a comparative analysis of their morphological and biochemical properties with broiler subcutaneous PAs. As a result, broiler FAPs share several features with FAPs isolated from humans or mice. Moreover, adipogenic differentiation of broiler FAPs was regulated in a comparable manner to that of PAs. However, broiler FAPs exhibited differences in fatty acid compositions and transcriptomic changes compared to PAs. These findings provide further insights into the biology of broiler FAPs and contribute to the understanding of the mechanisms underlying broiler myopathies.

The isolated broiler FAPs exhibited a morphological difference with a larger and more spread-out shape compared to myogenic cells, similar to the previously reported morphology of human and mouse FAPs. Broiler FAPs were PDGFR α -positive and negative for CD45, CD56, and pax7, matching the immunotype of human and mouse FAPs (Joe et al., 2010; Uezumi et al., 2010; Arrighi et al., 2015; Uezumi et al., 2014). The ability of broiler FAPs to differentiate into lipid-filled cells and collagen-1-producing cells suggests that they could be a cellular source of fibrofatty tissue in wooden breast-affected muscle

tissue in broilers. However, additional *in vivo* experiments are necessary for accurate confirmation.

The murine 3T3-L1 cell line is a commonly used *in vitro* model for studying adipogenic differentiation (Cave et al., 2019). This cell line is induced to undergo adipogenic differentiation by supplementing media with differentiation cocktails that typically consists of isobutylmethylxanthine, dexamethasone, and insulin, and sometimes thiazolidinediones (TZDs), such as rosiglitazone (Kokta et al., 2008; Zebisch et al., 2012; Cheng et al., 2016). In contrast, broiler PAs can be induced to differentiate by the inclusion of CS, with or without additional fatty acids. The effects of these various methods to induce adipogenic differentiation were compared between FAPs and PAs. The effects of each formulation on adipogenic differentiation were comparable in both cell types, indicating that the induction of adipogenic differentiation of progenitors derived from muscle and adipose tissues is similar. Each of the additions to CS significantly increased lipid droplet size in both cell types but did not affect total lipid accumulation per cell. Intriguingly, fatty acid mixtures containing oleic and linoleic acids enhanced the adipogenic differentiation of broiler FAPs and PAs in the media containing FBS. However, in the presence of FBS, the differentiation cocktails and TZDs were not effective inducers for adipogenic differentiation in both cell types (data not shown), indicating that fatty acids, particularly oleic and linoleic acids, are important inducers for chicken adipogenic differentiation (Matsubara et al., 2005). To further investigate these differences between FBS and CS, the fatty acid composition of each serum was analyzed. Chicken serum had a higher content of n-6 polyunsaturated fatty acids and a higher n-6/n-3 fatty acid ratio (about 3-fold higher) than FBS (data not shown). Consistent with this result, several studies demonstrated that a reduced n-6/n-3 fatty acid ratio decreased adipogenesis both *in vivo* and *in vitro* (An et al., 2012; Rudolph et al., 2018; Torres-Castillo et al., 2018; Kim and Voy, 2021). Taken together, these findings suggest that the n-6/n-3 fatty acid ratio, as well as the type of fatty acid, plays a critical role in chicken adipogenesis. In the comparative analysis between FAPs and PAs, the morphology of the derived adipocytes from both cell types was similar. However, FAPs had a lower lipid content and smaller lipid droplet size than PAs, which is consistent with previous findings (Arrighi et al., 2015). Once lipid droplets are assembled, they can grow by synthesizing triglycerides using excess intracellular fatty acids or by fusing with other lipid droplets (Olzmann et al., 2019). Diacylglycerol O-Acyltransferase 2 (DGAT2) mainly catalyzes the synthesis of triglycerides. In targeted RNA sequencing data, PAs displayed higher gene expression of DGAT2 than FAPs across adipogenic differentiation (data not shown),

supporting the difference in lipid droplet size. In addition, previous studies showed that adipogenic, lipogenic, and lipolytic-related genes are expressed at lower levels in intramuscular adipocytes compared to subcutaneous adipocytes in beef cattle and pigs. This difference in gene expression may be due to differences in the size of lipid droplets (Gardan et al., 2006; Gondret et al., 2008; Zhou et al., 2010; Pickworth et al., 2011; Wang et al., 2013; Chu et al., 2017; Huang et al., 2017).

Fatty acids can act as signaling molecules that regulate gene expression and cellular processes, such as insulin sensitivity and inflammation, in adipocytes (Ramel et al., 2008; Andersen et al., 2008; Samovski et al., 2023). The FAME analysis revealed that overall fatty acid composition of FAPs and PAs was similar, but there were differences in the percentage of unsaturated fatty acids (C20-24), long- and very long chain saturated fatty acids. The unsaturated, long chain fatty acids are produced from palmitic acid (C16:0), which is synthesized *de novo* by fatty acid synthase. Very long-chain polyunsaturated fatty acids are produced from dietary precursors, such as C18:2 and C18:3. The elongation and desaturation processes, which involve gene expression of the ELOVL, SCD, and FADS families, contribute to the synthesis of these fatty acids (Guillou et al., 2010; Tosi et al., 2014). In this study, indices of enzyme activity, specifically D5D and D6D, as well as gene expression of FADS6 and ELOVL1, 4, and 7, were significantly higher in PAs compared to FAPs (Data not shown). Delta-5 desaturase and delta-6 desaturase are encoded by FADS1 and FADS2 (Tosi et al., 2014). Additionally, the level of DHA and EPA in FAPs was significantly lower than in PAs. The observed differences in enzyme activity, gene expression, and fatty acid level can have a significant effect on the properties of intracellular lipid membranes, regulating signal transduction pathways (Dyall et al., 2015; Mason et al., 2016; Sherratt et al., 2018; McGlory et al., 2019; Hu et al., 2022).

Although adipose tissue accumulation in broilers has been extensively studied, the adipogenic differentiation program of broiler FAPs as a cellular source of fibrofatty infiltration in myopathies has not been investigated. In this study, we aimed to investigate the differences in the molecular regulatory mechanisms related to adipogenic differentiation between FAPs and subcutaneous PAs. Targeted RNA sequencing provided differences in a subset of genes of interest between the two cell types (Table 3). Several genes related to white adipocyte marker (TCF21, dermatopontin), glucose and fatty acid metabolism (cytochrome P450 family 24 subfamily A member 1, fatty acid binding protein 1, glutathione peroxidase 4, glycerol-phosphate dehydrogenase 1-like 2, insulin receptor substrate 1, prostaglandin D2 synthase, apelin, apelin receptor 2, pyruvate dehydrogenase

kinase 2, and pyruvate dehydrogenase kinase 4), fatty acid transport and oxidation (PR domain containing 16, fatty acid binding protein 1, perilipin 2, ribosomal protein S6 kinase B1, extracellular fatty acid binding protein, and insulin receptor substrate 1), and angiogenesis (angiotensin I converting enzyme, angiotensin II receptor type 1, angiotensin II receptor type 2, platelet derived growth factor receptor alpha, transcription factor 21, transforming growth factor beta receptor 2, and vascular endothelial growth factor D) were commonly upregulated during adipogenic differentiation in both FAPs and PAs. This provides insights into the molecular mechanisms underlying broiler intramuscular adipose tissue development and interventions to prevent metabolic disorders associated with aberrant FAPs adipogenesis.

Despite these similarities, several genes were robustly upregulated during differentiation of PAs, with no change in expression in FAPs. These include CCAAT enhancer binding protein alpha, CD24, Wnt family member 4, angiotensinogen, Preadipocyte factor 1, basigin, cannabinoid receptor 1, DGAT2, interleukin 10, myocyte enhancer factor 2C, and sclerostin. On the other hand, C-C motif chemokine ligand 20, FTO alpha-ketoglutarate dependent dioxygenase, LDL receptor related protein 1 (LRP1), PPARG coactivator 1 alpha, Wnt family member 10A, homeobox C9, nudix hydrolase 6, period circadian regulator 3, secreted frizzled related protein 5, and solute carrier family 1 member 3 were upregulated only during adipogenic differentiation in FAPs. This subset of genes likely plays crucial roles in fatty acid metabolism, lipid metabolism, and differentiation in PAs, not in FAPs.

Intriguingly, during adipogenic differentiation, myocardin and myogenin were upregulated in PAs but downregulated in FAPs, and Hey1 and CD34 were upregulated in FAPs but downregulated in PAs. Myocardin and myogenin are transcription factors that play crucial roles in muscle development and differentiation. The upregulation of myocardin and myogenin in PAs suggests a potential role in regulating adipocyte differentiation, which may be different from their known functions in muscle development. However, their functional role in adipogenesis has not yet been investigated. The downregulation of these factors in FAPs during adipogenesis may also indicate differences in the regulatory mechanisms underlying adipocyte differentiation in these two cell types. MKL1, a member of the myocardin family, has an anti-adipogenic effect on non-muscle cells, and its deficiency enhances white adipose tissue browning (McDonald et al., 2015). Nevertheless, some MKL1 targets, including DGAT2 and LRP1, promote adipogenic differentiation (Svärd et al., 2016). While myogenin is a well-known marker for myogenic differentiation, several studies

have reported its expression in adipocytes and FAPs (Timmons et al., 2006; Biferali et al., 2021; Wang et al., 2021). These unexpected expression patterns during adipogenesis raise interesting questions about their potential roles and functions.

Hey1, a Notch signaling target gene, is critical for adipogenesis. Hey1 expression is increased during murine preadipocyte differentiation, and its deficiency or enforced expression led to impaired adipogenic differentiation by decreasing expression of key activators of adipogenesis such as C/EBP α , PPAR γ , and FABP4 (Gerin et al., 2009; Scroyen et al., 2015). CD34, a hematopoietic stem cell marker, is expressed in adipose mesenchymal stem/stromal cells (APCs) in adipose tissue, but it is lost during cell expansion in vitro (Scherberich et al., 2013). Adipocytes derived from APCs with high CD34 expression show higher rates of lipolysis and fatty acid uptake compared to adipocytes derived from no CD34 expression which exhibit beige-like adipocyte properties (Raajendiran et al., 2019).

In conclusion, this study showed the differentiation capacity and properties of broiler FAPs compared to subcutaneous PAs. Fibroadipogenic progenitors have a comparable differentiation capacity to PAs but exhibit differences in morphology and biochemistry, suggesting differences in lipid metabolism. Transcriptomic analysis also revealed differences in gene expression profiles between the two cell types during differentiation, which may reflect their distinct origins and functions. These findings provide valuable insights into the biological properties of broiler FAPs and their potential as a cellular source in the development of wooden breast myopathy.

Appendix 3

Table 2. Composition (%) of fatty acid methyl esters in broiler FAPs and PAs

Item ¹	General name	% of total content		P-value
		PAs	FAPs	
C6:0	Caprioic acid	0.000	0.017	0.122
C8:0	Caprylic acid	0.000	0.015	<0.001
C10:0	Capric acid	0.003	0.014	<0.001
C11:0	Undecylic acid	0.000	0.003	0.029
C12:0	Lauric acid	0.092	0.094	0.674
C13:0	Tridecylic acid	0.004	0.005	0.281
C14:0	Myristic acid	0.425	0.423	0.872
C14:1n-5	Myristoleic acid	0.055	0.047	<0.001
C15:0	Pentadecylic acid	0.116	0.121	0.182
C15:1n-5	cis-10-pentadecanoic acid	0.008	0.008	0.944
C16:0	Palmitic acid	30.38	30.57	0.731
C16:1n-7	Palmitoleic acid	2.828	2.788	0.412
C17:0	Margaric acid	0.177	0.186	0.254
C17:1n-7	cis-10-heptadecenoic acid	0.083	0.084	0.619
C18:0	Stearic acid	18.227	19.048	0.151
C18:1n-9	Oleic acid	26.96	26.46	0.360
C18:2n-6	Linoleic acid	14.74	14.07	0.155
C18:3n-3	α -Linolenic acid	0.153	0.132	<0.001
C18:3n-6	γ -Linolenic acid	0.827	1.236	<0.001
C20:0	Arachidic acid	0.53	0.110	<0.001
C20:1n-9	Cetoleic acid	0.203	0.208	0.382
C20:2n-6	Eicosadienoic acid	0.159	0.152	0.053
C20:3n-3	Eicosatrienoic acid	0.396	0.334	<0.001
C20:3n-6	Dihomo- γ -Linolenic acid	0.596	0.542	0.008
C20:4n-6	Arachidonic acid	2.998	2.910	0.351
C20:5n-3	Eicosapentaenoic acid	0.119	0.098	0.005
C21:0	Heneicosylic acid	0.002	0.003	0.002
C22:0	Behenic acid	0.011	0.060	<0.001
C22:1n-9	Erucic acid	0.182	0.044	0.011
C22:2n-6	Docosadienoic acid	0.011	0.007	0.046
C22:5n-3	Docosapentaenoic acid	0.096	0.090	0.119
C22:5n-6	Osbond acid	0.037	0.033	0.038
C22:6n-3	Docosahexaenoic acid	0.058	0.050	0.032
C23:0	Tricosylic acid	0.000	0.001	0.007
C24:0	Lignoceric acid	0.003	0.040	<0.001
Σ SFA		49.50	50.71	0.102
Σ MUFA		30.32	29.64	0.233
Σ PUFA		20.18	19.65	0.281
Σ n-6		19.36	18.95	0.388
Σ n-3		0.822	0.705	<0.001
Σ n-6/ Σ n-3		23.57	26.94	0.002

Table 2. (Continued)

Activity index²	General name	PAs	FAPs	P-value
SCD	Stearoyl-CoA desaturase	0.613	0.590	0.228
D5D	Delta-5 desaturase	5.03	5.37	0.002
D6D	Delta-6 desaturase	0.056	0.088	<0.001
DNL	De novo lipogenesis	2.069	2.175	0.180
EL	Elongation	0.601	0.624	0.321

Values are expressed as mean ($n = 6$).

* $P < 0.05$ by Student's t-test.

¹ % of total is the relative percentage of fatty acid methyl esters measured in each sample.

² Enzyme activities were calculated using the following equations: SCD; 16:1n-7+18:1n-9/16:0+18:0, D5D; 20:4n-6/20:3n-6, D6D; 18:3n-6/18:2n-6, DNL; 16:0/18:2n-6, EL; 18:0/16:0.

Abbreviations: FAPs, fibroadipogenic progenitors; PAs, preadipocytes; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. DEGs (FDR < 0.05) during adipogenic differentiation in FAPs and PAs

Gene Symbol	Gene Name	Gene Identifier	Fold change (96h vs 0h)	
			PAs	FAPs
Upregulated DEGs in both FAPs and PAs				
ACE	angiotensin I converting enzyme	ENSGALG00010023369	7.40	6.33
AGTR1	angiotensin II receptor type 1	ENSGALG00010017056	8.94	25.02
ALPL	alkaline phosphatase, liver/bone/kidney	ENSGALG00010021115	2.73	2.56
APLNR2	G protein-coupled receptor 25	ENSGALG00010026683	2.61	5.88
BMP15	bone morphogenetic protein 15	ENSGALG00010016722	2.23	3.10
CCL5	C-C motif chemokine ligand	ENSGALG00010029947	6.29	6.01
CCR6	C-C motif chemokine receptor 6	ENSGALG00010006499	5.84	2.08
CHST12	carbohydrate sulfotransferase 12	ENSGALG00010020032	3.52	3.11
DPT	dermatopontin	ENSGALG00010005369	4.94	4.07
EXFABP	extracellular fatty acid-binding protein	ENSGALG00010028912	25.98	7.32
FABP1	fatty acid binding protein 1	ENSGALG00010016147	4.60	2.57
FBP2	fructose-bisphosphatase 2	ENSGALG00010010323	7.11	2.87
HRH2	histamine receptor H2	ENSGALG00010014576	5.12	5.71
IRS1	insulin receptor substrate 1	ENSGALG00010023620	1.98	3.75
NPY	neuropeptide Y	ENSGALG00010002375	3.05	2.30
PDK2	Pyruvate dehydrogenase kinase 2	ENSGALG00010025274	2.95	2.59
PLIN2	perilipin 2	ENSGALG00010016168	2.83	2.33
PRDM16	PR/SET domain 16	ENSGALG00010019648	4.90	3.18
PRKG2	protein kinase, cGMP-dependent, type II	ENSGALG00010003198	2.47	4.09
PTGDS	prostaglandin D2 synthase	ENSGALG00010028450	11.47	6.75
SLC25A34	solute carrier family 25 member 34	ENSGALG00010021797	3.53	2.81
SPON2	spondin 2	ENSGALG00010017676	4.92	1.96
SSTR2	somatostatin receptor 2	ENSGALG00010029470	6.95	5.39
TGFBR2	transforming growth factor beta receptor 2	ENSGALG00010002281	4.49	6.27
TRPV6	transient receptor potential cation channel, subfamily V, member 6	ENSGALG00010021482	5.94	2.28
VEGFD	vascular endothelial growth factor D	ENSGALG00010001454	3.46	11.13
ABCA1	ATP-binding cassette, sub-family A, member 1	ENSGALG00010014122	2.81	1.84
AGTR2	angiotensin II receptor type 2	ENSGALG00010018860	2.55	1.60
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	ENSGALG00010001525	2.02	1.90
GPD1L2	glycerol-3-phosphate dehydrogenase 1-like 2	ENSGALG00010020525	6.03	1.85
GPX4	glutathione peroxidase 4	ENSGALG00010027996	2.74	1.65
IL15	interleukin 15	ENSGALG00010009362	2.69	1.89
MMP16	matrix metallopeptidase 16	ENSGALG00010012390	1.95	1.67
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	ENSGALG00010013621	2.43	1.63
CD14	CD14 molecule	ENSGALG00010011659	1.82	4.32

Table 3. (Continued)

CYP24A1	cytochrome P450 family 24 subfamily A member 1	ENSGALG00010015154	1.66	6.55
MMP9	matrix metalloproteinase 9	ENSGALG00010015061	1.86	2.71
PCNX4	pecanex homolog 4	ENSGALG00010016943	1.84	3.49
PDK4	pyruvate dehydrogenase kinase 4	ENSGALG00010002741	1.51	2.17
RPS6KB1	ribosomal protein S6 kinase B1	ENSGALG00010029283	1.88	2.19
TCF21	transcription factor 21	ENSGALG00010006187	1.44	3.41
WNT5B	Wnt family member 5B	ENSGALG00010013899	1.63	4.96
Upregulated DEGs only in PAs				
CNR1	cannabinoid receptor 1	ENSGALG00010003541	3.79	1.80
CD24	CD24 molecule	ENSGALG00010012582	2.18	1.94
MEF2C	myocyte enhancer factor 2C	ENSGALG00010007430	3.41	1.59
DLK1	delta like non-canonical Notch ligand 1	ENSGALG00010016760	2.61	1.70
BSG	basigin	ENSGALG00010027468	2.35	1.42
AGT	angiotensinogen	ENSGALG00010007759	5.55	1.97
IL10	interleukin 10	ENSGALG00010027138	3.38	1.54
DGAT2	diacylglycerol O-acyltransferase 2	ENSGALG00010003035	2.67	1.27
CEBPA	CCAAT/enhancer binding protein alpha	ENSGALG00010025039	1.99	1.40
WNT4	Wnt family member 4	ENSGALG00015025065	8.90	1.25
SOST	sclerostin	ENSGALG00010024290	4.42	1.03
Upregulated DEGs only in FAPs				
PER3	period circadian clock 3	ENSGALG00010018796	1.36	2.96
LRP1	LDL receptor related protein 1	ENSGALG00010025368	1.33	2.69
NUDT6	nudix hydrolase 6	ENSGALG00010004131	1.32	2.16
PPARGC1A	PPARG coactivator 1 alpha	ENSGALG00010010006	1.44	2.55
SLC1A3	solute carrier family 1 member 3	ENSGALG00010013632	1.37	12.19
WNT10A	Wnt family member 10A	ENSGALG00010021151	0.73	2.89
CCL20	C-C motif chemokine ligand 20	ENSGALG00010023872	1.20	9.36
SFRP5	secreted frizzled related protein 5	ENSGALG00010017808	1.14	9.40
FTO	FTO, alpha-ketoglutarate dependent dioxygenase	ENSGALG00010021200	0.98	1.96
HOXC9	homeobox C9	ENSGALG00010025958	1.01	3.25
DEGs with Opposite Expression Patterns in FAPs and PAs				
MYOCD	myocardin	ENSGALG00010029743	2.25	0.40
MYOG	myogenin	ENSGALG00010025215	2.31	0.05
HEY1	hes related family bHLH transcription factor with YRPW motif 1	ENSGALG00010003330	0.55	4.49
CD34	CD34 molecule	ENSGALG00010027183	0.42	2.06

Abbreviations: DEGs; differentially expressed genes.

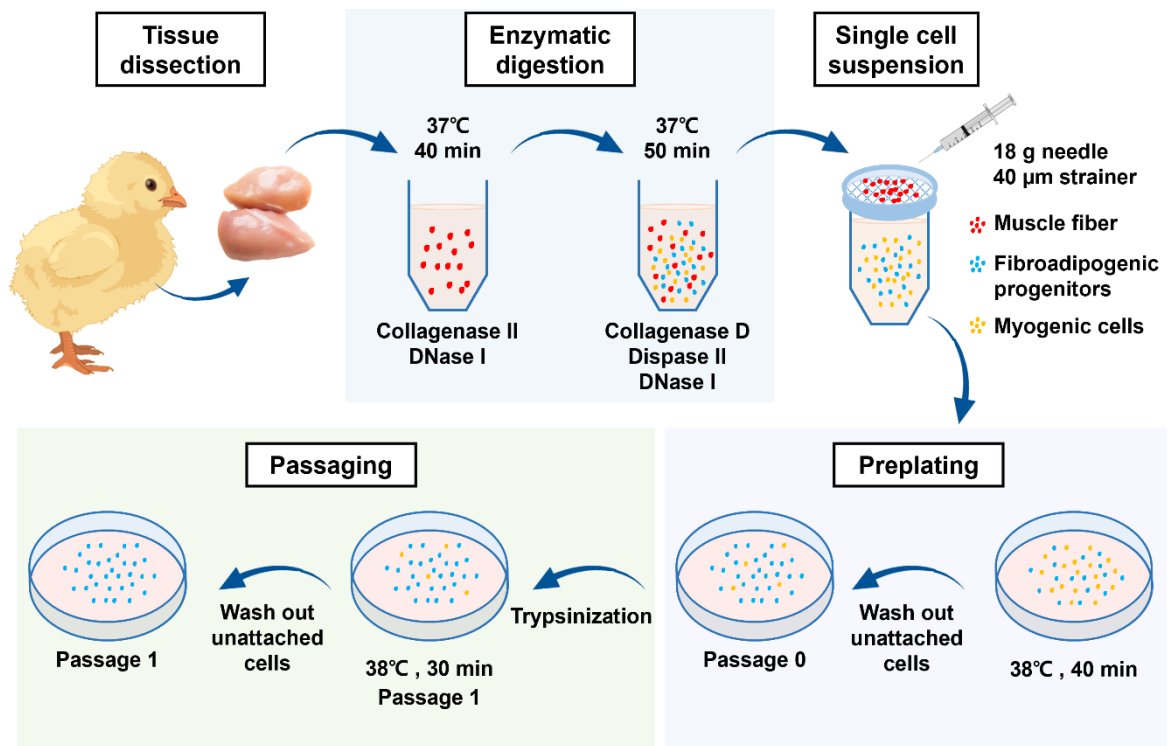


Figure 4. Overview of primary fibroadipogenic progenitor isolation from pectoralis major in broiler chick

Schematic diagram detailed in methods. Single-cell suspension is processed through two-step enzymatic digestion. Fibroadipogenic progenitors are purified by pre-plating techniques based on the adhesion properties.

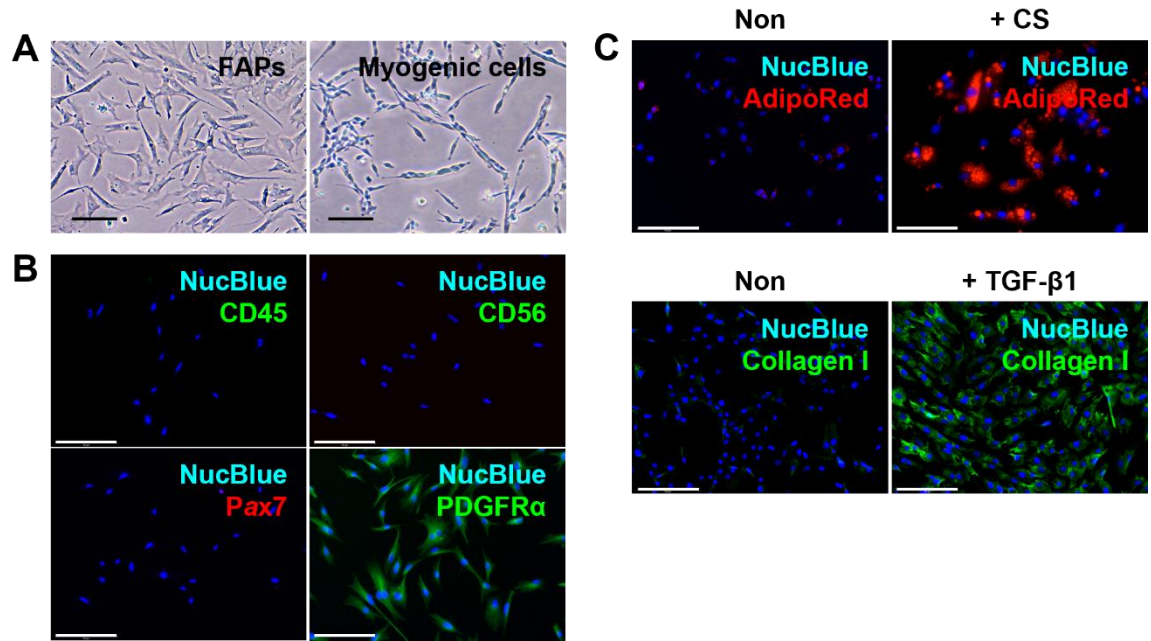


Figure 5. Cell-surface immunophenotype and differentiation potential of broiler fibroadipogenic progenitors

(A) phase contrast microscopic images of broiler fibroadipogenic progenitors (FAPs) (left) and myogenic cells (right). Scales bar = 100 μ m (B) Immunostained broiler FAPs with CD45, CD56, Pax7, and PDGFR α . Scales bar = 150 μ m (C) Differentiation of broiler FAPs into adipocytes and type 1 collagen-producing cells, respectively. Scales bar = 150 μ m.

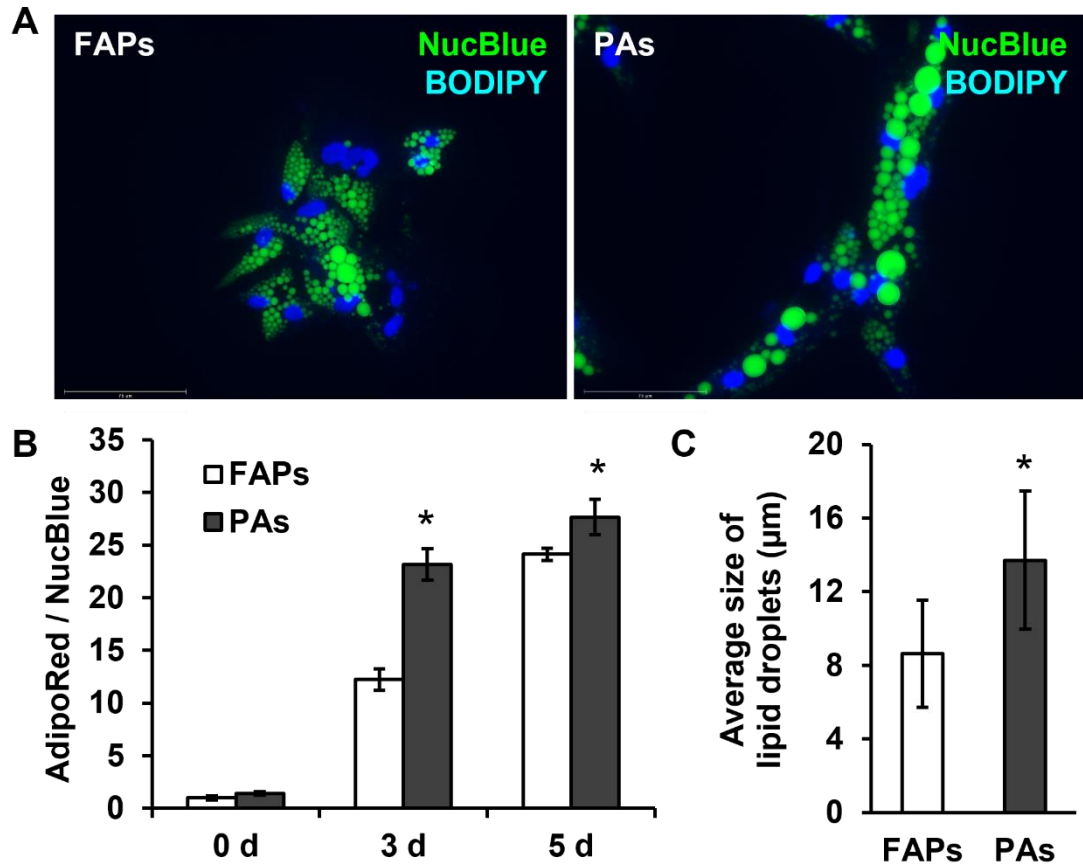


Figure 6. Comparison of adipogenic differentiation in fibroadipogenic progenitors and preadipocytes

(A) representative images of lipid staining (green) and DNA staining (blue) (B) Lipid accumulation was measured by detecting fluorescent probes within the cells and normalized to DNA content (C) average size of lipid droplets in fibroadipogenic progenitors (FAPs) and preadipocytes (PAs). Values are shown as the mean $\pm$ SD ($n = 3$). * $P < 0.05$.

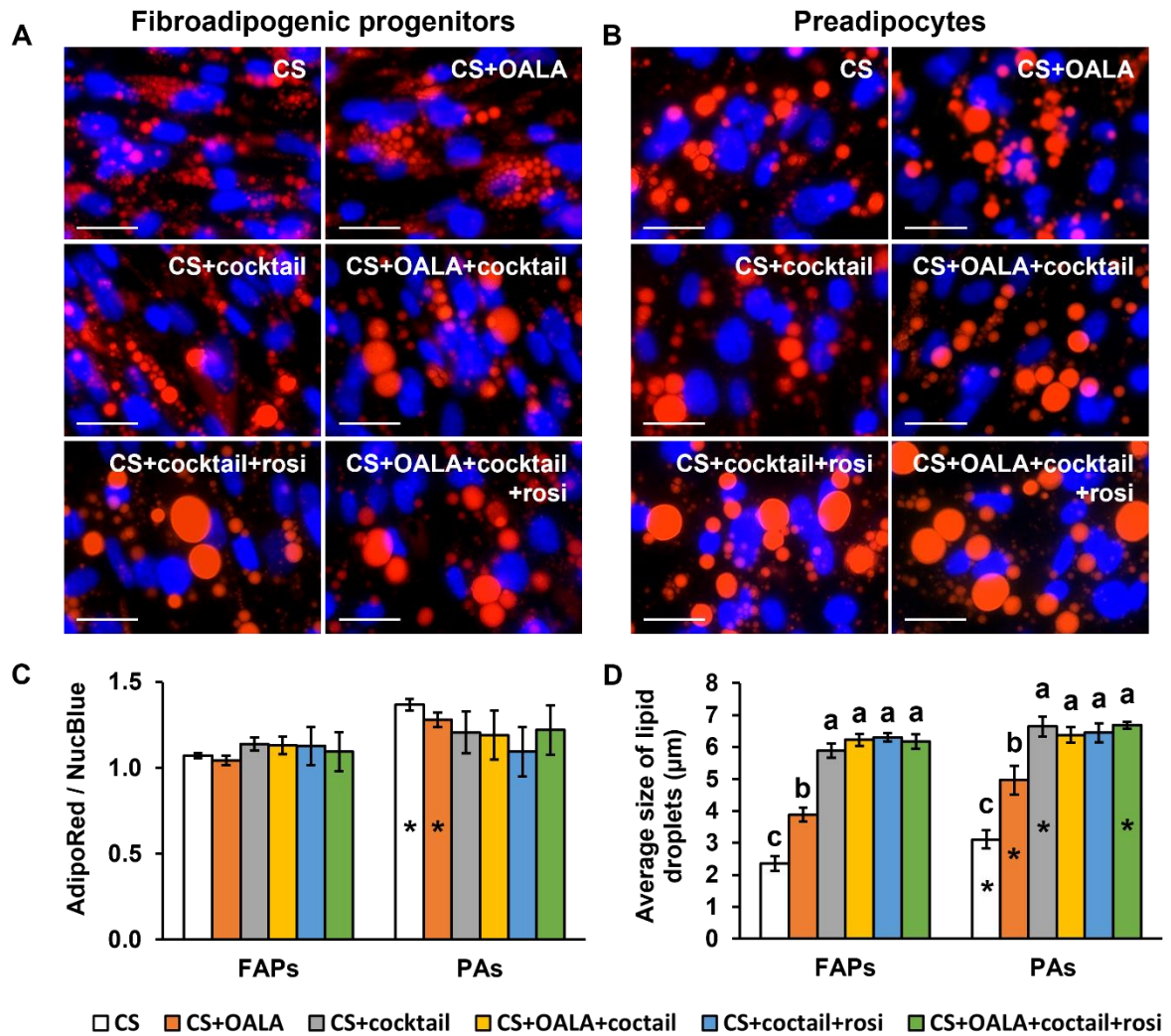


Figure 7. Supplemental effects of fatty acid mixtures, differentiation cocktails (isobutylmethylxanthine, dexamethasone, and insulin), and rosiglitazone on induction of adipogenic differentiation in fibroadipogenic progenitors and preadipocytes

(A) representative images of lipid staining (red) and DNA staining (blue) of broiler fibroadipogenic progenitors (FAPs) (B) preadipocytes (PAs) (C) lipid accumulation was normalized to DNA content (D) average size of lipid droplets in FAPs and PAs. Values are shown as the mean $\pm$ SD ($n = 3$). Asterisks indicate significant difference between FAPs and PAs: $*P < 0.05$. The different lowercase letters (a, b, or c) above columns indicate significant differences among various treatments in FAPs or PAs ($P < 0.05$).

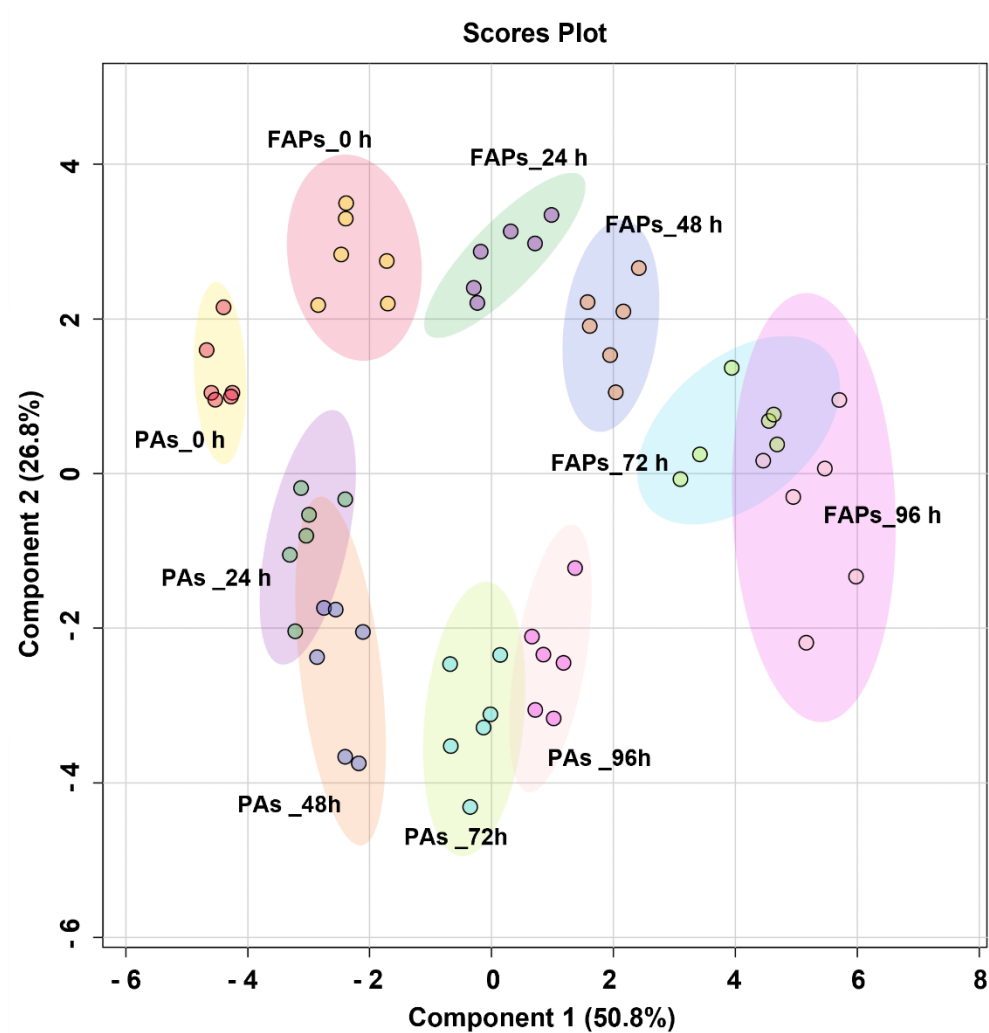


Figure 8. PLS-DA visualization of sample clustering in broiler fibroadipogenic progenitors and preadipocytes across differentiation

Data from each fibroadipogenic progenitors (FAPs) and preadipocytes (PAs) sample is encoded by color. Analyses were performed using Metaboanalyst (metaboanalyst.ca) using default settings. Statistical significance of the sample clustering was evaluated with a permutation test (1,000 times) ($P < 0.001$).

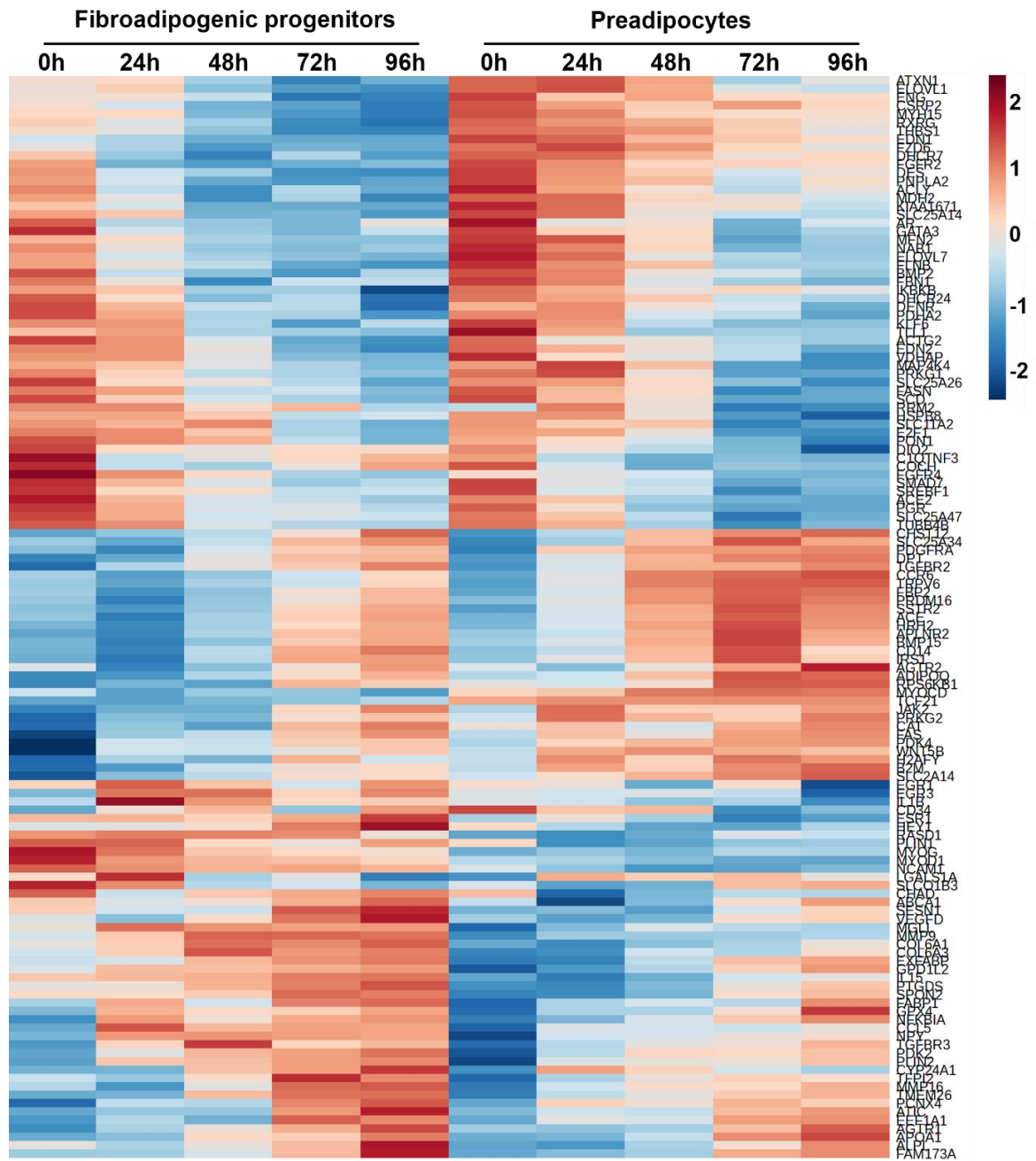


Figure 9. Heatmap of 129 genes that were commonly regulated in both broiler fibroadipogenic progenitors and preadipocytes across differentiation

Analyses were performed using Metaboanalyst (metaboanalyst.ca) using default settings. Red indicates up-regulation, and blue indicates down-regulation. FDR $P < 0.05$. ($n = 6$).

CHAPTER III: HYPOXIA PROMOTES PROLIFERATION AND INHIBITS MYOGENESIS IN BROILER SATELLITE CELLS

This manuscript is being prepared for publication with co-authors Minjeong Kim, Presley Dowker, Ahmed Bettaieb, and Brynn H. Voy. for Poultry Science.

Abstract

Broiler breast muscle myopathies are characterized by impaired satellite cell-mediated repair, and localized tissue hypoxia and dysregulate oxygen homeostasis have been implicated as contributing factors. Here, I demonstrated that hypoxia culture conditions enhanced proliferation by upregulating Pax7 expression, along with activation of cell-cycle molecules. In addition, hypoxia significantly inhibited myogenic potential of broiler SCs, with decreased fusion index and suppressed myotube formation. However, reoxygenation after hypoxia partially rescued both cell proliferation and myogenic potential. Interestingly, hypoxia promoted the accumulation of lipid droplets in myotubes. Western blotting showed that hypoxia diminished myogenin expression, activated AMPK, upregulated proliferation marker, and increased molecular markers of cellular stress. Targeted RNAseq was used to profile expression of a panel of 522 genes that broadly query proliferation, myogenesis, and hypoxia. Thirteen genes were highly regulated across 48 h hypoxia exposure. Altogether, these data support that oxygen levels play a central role in regulating cellular proliferation and differentiation in broiler SCs. Given the effects of hypoxia on broiler SCs, I predict that hypoxia potentially inhibits satellite cell-mediated muscle repair and increases lipid accumulation in breast muscles affected by broiler breast myopathies.

Introduction

Intensive genetic selection for high growth rate, breast muscle yield, and feed efficiency in modern broilers has proven to be a double-edged sword. Broiler breast myopathies (e.g., wooden breast) arise from the extremely rapid growth of breast muscle and compromise meat quality in ways that impose significant losses to the broiler industry. Myopathic lesions are histopathologically characterized by increased myofiber degeneration, reduced SCs-mediated regeneration, and fibrofatty tissue infiltration (Velleman et al., 2017; Papah et al., 2017; Sihvo et al., 2018). Myopathies are thought to begin with damage to muscle fibers that results from cellular and tissue consequences of extremely rapid growth, which is specific to the pectoralis major muscle. However, the etiology of broiler breast myopathies is unclear.

Skeletal muscle is primed to repair itself from both physiological damages, such as that induced by demanding exercise, as well as from injury and other more significant

insults. The ability to repair comes from SCs, muscle stem cells that align with muscle fibers and lie just under the sarcolemma. Upon muscle injury, SCs proliferates and differentiates to provide new muscle fibers to replace sites of damage. Disruption of SCs' ability to fulfill this role is implicated in the etiology of myopathies in broilers. Previous studies revealed that both proliferation and myogenic differentiation of SCs are compromised in wooden breast-affected muscles (Daughtry et al., 2017; Ferreira et al., 2020). Therefore, it is imperative to investigate key factors influencing the proliferation and myogenic differentiation of broiler SCs.

Localized tissue hypoxia in pectoralis major of broilers due to extremely rapid growth is linked to failure of efficient muscle repair and development of breast muscle myopathies. Hypoxia is thought to develop soon after hatch when muscle growth outpaces capillary development, resulting in diminished tissue perfusion. This hypothesis leading to the development of broiler myopathies has been well supported by microscopic observations, gene expression, and metabolomes. Wooden breast-affected muscle exhibited a higher myofiber area per vessel than unaffected muscles and increased expression of oxidative stress response-related genes and biomarkers (Mutryn et al., 2015; Abasht et al., 2016; Sihvo et al., 2018). Increased expression of hypoxia-inducible factor 1 alpha (HIF-1 α), a transcription factor that senses hypoxia and coordinates cellular adaptation, has been reported in breast muscle of broilers affected with myopathies (Mutryn et al., 2015; Papah et al., 2018; Greene et al., 2019; Malila et al., 2019; Emami et al., 2021).

Transient hypoxia, a normal physiological stimulus in skeletal muscle, can occur due to reduced oxygen delivery due to muscle injury, damage, or chronic inflammation affecting tissue capillaries (Hotta et al., 2018; Drouin et al., 2019). During intense exercise, transient hypoxia in skeletal muscle is also caused by a marked decrease in oxygen availability due to increased energy demands for muscle contraction (Skovgaard et al., 2009). Exercise-induced transient hypoxia can trigger increased blood flow and angiogenesis (formation of new blood vessels), and lead to muscle hypertrophy or muscle mass reduction, depending on the degree of oxygen deprivation (Chaillou, 2018; Lemieux and Birot, 2021). In broilers, however, localized hypoxia within the pectoralis major muscle is likely to persist and may even escalate as the muscle undergoes progressive hypertrophy until harvest at market age. This indicates that this localized tissue hypoxia in breast muscle may contribute to initial myofiber damage by promoting oxidative stress and disrupting tightly coordinated steps in myogenesis that are necessary for healthy muscle repair. Previous studies demonstrated that hypoxic environments are crucial for SCs-mediated muscle regeneration at the site of injured

skeletal muscle (Leroux et al., 2010; Chaillou and Lanner, 2016). Recently, hypoxia downregulated expression of myogenic proteins in SCs (Emami et al., 2021; Elashry et al., 2022). Collectively, these results suggest that hypoxia might play important roles in SCs-mediated muscle growth and regeneration and are a key factor in the development of broiler breast myopathies. Therefore, it is essential to understand the potential contribution of hypoxia to the development of broiler breast myopathies. Here, we investigated the effects of hypoxia on the proliferation and differentiation in broiler SCs.

Materials and methods

Animal and housing

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee-Knoxville (protocol no. 2164). Day-old male Cobb 700 chicks were purchased from a local hatchery and raised in a pen filled with pine shavings and provided water and feed ad libitum. Chicks were euthanized by CO₂ asphyxiation at age 5, and pectoralis major muscle was collected and harvested into sterile PBS for subsequent isolation of SCs.

Isolation and culture of satellite cells

Primary SCs were isolated as previously described from chicks (Jung et al., 2022). Briefly, approximately 2 g of breast muscle was finely minced and subject to a two-step digestion, using first collagenase II (Sigma, St. Louis, MO) and DNase I (Roche, Indianapolis, IN), and then collagenase D (Roche, Indianapolis, IN), Dispase II (Sigma, St. Louis, MO), and DNase I. Final digest was filtered through 40-µm cell strainers (SPL Life Sciences, Pocheon, Korea) and centrifuged to collect cells. Cell pellets were then resuspended in DMEM/F12 containing 10% fetal bovine serum, 100 IU/mL penicillin/streptomycin, and 2.5 µg/mL amphotericin B. Primary broiler SCs were purified by preplating, and cultured in growth media (**GM**) containing 20% horse serum, 10% fetal bovine serum, 1% chicken embryo extract, 2.5 ng/mL basic fibroblast growth factor, and 100 IU/mL penicillin/streptomycin in DMEM/F10 at 38.0°C in a humidified incubator, with a 5% CO₂/95% O₂ (designated as normoxia), unless otherwise specified. Where noted, myogenic differentiation was induced by replacing GM with differentiation media (DMEM/F12 containing 10% horse serum and 2% chicken embryo extract) after washing cells with 1X PBS.

Induction of hypoxia

Hypoxic culture conditions were created using a hypoxia incubator chamber (Stemcell, Cambridge, MA, USA) in which the gas environment consisted of 1% O₂, 5% CO₂, and 94% N₂. The chamber was flushed with the custom gas mixture for one minute at 25 psi prior to transferring cells from normoxic growth conditions. To model the effects of hypoxia on myogenic differentiation, SCs were transferred to the hypoxia chamber at ~ 70% confluence and cultured under these conditions in differentiation media for 48 h. To determine if effects of hypoxic culture could be rescued by a return to normoxia, SCs were maintained under hypoxia for 24 h and then transferred to normoxic conditions for an additional 24 h.

Proliferation assays

Effects of hypoxia and rescue on SCs proliferation were assessed directly using a Click-iT™ EdU proliferation assay (Thermo). Satellite cells were seeded at 5 x 10³ cells/well into collagen type I-coated 96 well plates, and 25 μM EdU was added to each well to a final concentration of 5 μM. Cells were incubated for 48 h under normoxia, hypoxia, or rescue condition, after which proliferation was assessed following the manufacturer's protocol. Fluorescence intensity was measured with excitation maximum of 568 nm and emission maximum of 585 nm using a BioTek Synergy H1 Multimode Reader (Agilent, Santa Clara, CA).

HEMA3 staining and myogenic evaluation

Fused myonuclei was assessed by visually staining SCs with HEMA3 (Thermo Fisher Scientific, Waltham, MA), a shelf-stable and ready to use stain that was shown to robustly stain myotubes (Levitt et al., 2019). Cells were rinsed twice with 1X PBS and fixed in methanol at RT for 10 min. Methanol-fixed cells were dried in a laminar flow biosafety cabinet and incubated with HEMA 3 solution I at RT for 5 min. Cells were washed twice in distilled water for 5 min and incubated in HEMA3 solution II for 5 min. Stain was removed and cells were washed twice in distilled water prior to imaging.

Lipid droplet staining

Lipid accumulation within myotubes was induced with the differentiation media supplemented with 94 ng/mL linoleic acid and 94 ng/mL oleic acid. Lipid droplets in myotubes were stained with BODIPY 493/503 (Thermo Fisher Scientific). Cultures were

washed in 1X PBS once and incubated with 1 μ M BODIPY 493/503 for 15 min at 38°C in a CO₂ incubator followed by fixed with 4% paraformaldehyde for 10 min at RT. Lipid droplets were visualized with an EVOS cell imaging system.

Immunocytochemistry

Proliferating and differentiating SCs were assessed by immunostaining with Pax7 (1:5, DHSB), MyoD (1:50, Santa Cruz Biotechnology), and myosin heavy chain (MF20, 1:10, DHSB). Cells were fixed in 4% paraformaldehyde at RT for 10 min and washed in 1X PBS three times. Fixed cells were permeabilized in 0.1% Triton X-100 (Sigma, St. Louis, MO) for 10 min at RT, washed once in 1X PBS, blocked in 2% bovine serum albumin (Sigma, St. Louis, MO) in 1X PBS for 30 min at RT, and then incubated with primary antibodies diluted in blocking buffer for at 4°C overnight in the dark. After incubation, the cells were washed three times with 1X PBS for 5 minutes each. Then, they were incubated with Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 594 conjugates (Thermo Fisher Scientific, Waltham, MA) for 2 h at RT. Then, cells were washed in 1X PBS three times for 5 min each followed by nuclear staining with Hoechst 33342 (diluted in 1X PBS) for 15 min. fluorescent images were captured with an EVOS cell imaging system. Cell cycle status of SCs was classified by counting nuclei expressing Pax7- or MyoD-positive or both with ImageJ (imagej.nih.gov).

Analysis of myogenesis

Myogenesis was evaluated by HEMA3 staining and immunostaining for MF20. The number of fused myonuclei per myotube were counted in HEMA3 images using ImageJ. MF20 images were used to measure fusion index which is calculated by counting the number of myonuclei within myosin heavy chain-positive myotubes divided by the total number of myonuclei.

Protein extraction and western blots

Satellite cell cultures were washed with 1X PBS and lysed on ice in RIPA buffer (150 mM NaCl, 10 mM Tris, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing freshly prepared protease (1mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 2 μ g/ml leupeptin) and phosphatase inhibitors (100 μ M sodium orthovanadate and 10mM p-nitrophenylphosphate), sonicated twice for 10 s each. Then, total protein concentration was measured using a bicinchoninic acid assay kit (Pierce

Chemical, Dallas, TX, USA) and the extracted proteins were quantitatively pooled by treatment. Then, proteins were loaded and transferred onto polyvinylidene fluoride membranes for western blot. Membranes were blocked with TBST containing 5% bovine serum albumin and 0.1% tween for 1 h at RT followed by probed with the commercially purchased primary antibodies (Table 4) for 1 h at RT. After washing blots three times in TBST for 5 min each, blots were incubated with secondary antibodies for 1 h at RT following 4 times wash cycles with TBST for 15 min each. Proteins were visualized using Luminata™ Western Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA).

Targeted RNA Sequencing

Myogenic differentiation of SCs was induced cultured under normoxia or hypoxia for 48 h. Cells were harvested immediately prior to induction of differentiation and at 12 h, 24 h, 36 h, and 48 h after differentiation induction for isolation of RNA. Total RNA was isolated using Direct-zol RNA Miniprep kit according to the manufacturer's instructions (Zymo Research, Irvine, California). RNA concentration and quality were determined by spectrophotometry and gel electrophoresis, respectively. Total RNA (500 ng) was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Prior to library preparation for targeted RNA sequencing, cDNA quality was confirmed based on PCR for Actb (F: 5'-ATG AAG CCC AGA GCA AAA GA-3', R: 5'-GCT GTG GTG GTG AAG CTG TA-3') and visualization of products using gel electrophoresis.

Targeted RNA sequencing was used to profile expression of 522 genes involved in proliferation, myogenesis, adipogenesis, cellular stress, and hypoxia, using methods previously described (Mihelic et al., 2020). Briefly, primers were designed based on the chicken refseq genome (Gallus_gallus-6.0/galGal6) using NCBI Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) to represent the consensus transcript for each gene and to yield products that spanned exon boundaries. Reads were aligned to the reference genome and read counts obtained using CLC Genomics Workbench (Qiagen, Germantown, MD).

Metabolic activity and reactive oxygen species measurement

Mitochondrial dehydrogenase activity was measured as an index of cellular metabolic activity using a commercial XTT assay (Biotinum, Hayward, CA). Briefly, equal number (5×10^3 cells/well) of SCs were seeded in GM on collagen type I-coated 96 well

plates for 24 h. Fresh XTT labeling mixture (50 μ L) was added to each well and the plates were incubated for 2 h at 38°C in CO₂ incubator. Absorbance for XTT (475 nm) was measured using a BioTek Epoch 2 Microplate Spectrophotometer (Agilent). Intracellular reactive oxygen species (ROS) production in both SCs and myotube was determined using the fluorogenic dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Figure 14). Cells were seeded in 24-well plates in DMEM/F12 containing 10% fetal bovine serum, 100 IU/mL penicillin/streptomycin followed by incubation at 38°C in a CO₂ incubator or the hypoxia chamber. After two days, cells were washed in DMEM/F12 without serum once and incubated in 1 μ M DCFDA for 30 min at 38°C in a CO₂ incubator or the hypoxia chamber. The dye was then removed, nuclei was stained with Hoechst 33342 for 15 min, cells were washed in DMEM/F12, and fluorescent intensity was measured by a BioTek Synergy H1 Multimode Reader set at an excitation wavelength of 485nm and emission wavelength of 528nm.

Statistical analysis

Statistical analyses for proliferation assay and immunocytochemistry were performed using PASW Statistics 18 Software (SPSS Inc., Chicago, IL). All data except for the targeted RNAseq were presented as mean with standard deviation and analyzed by one-way ANOVA with Tukey's test to assess the significant pairwise differences between treatments. Targeted RNA sequencing data were analyzed using EdgeR in R (version 4.1.3). Effects of multiple testing were controlled using false-discovery rate (FDR). FDR-adjusted p-values equal to or less than 0.05 were statistically significant. Statistical significance of the sample clustering was evaluated with a permutation test (1,000 times) ($P < 0.001$).

Results

Hypoxia stabilizes HIF-1 α and influences proliferation rate of satellite cells

Induction of hypoxia was confirmed using Western blot, based on stabilization of HIF-1 α protein. As shown in Figure 10, culture under hypoxic conditions (1% O₂) for 48 h increased HIF-1 α protein level. Compared to 1% O₂, HIF-1 α protein wasn't stabilized by 5% O₂ (data not shown). Protein abundance in the rescue group appeared to be intermediate between hypoxia and normoxia samples (Figure 10). Hypoxia significantly increased SCs proliferation vs. normoxia, based on measure of DNA replication (Figure 11). This effect was rescued by returning cells to normoxia after 24 h under hypoxic conditions.

Hypoxia alters behavior of satellite cells

Immunohistochemistry was used to determine if hypoxia and/or rescue alter the abundance of specific subpopulations of SCs, to assess the effects on SCs behavior and commitment toward myogenesis (Figure 12). Primary SCs was cultured under normoxia or hypoxia for 48 h and the relative expression of both Pax7 and MyoD was confirmed by immunocytochemistry and the percentage of SCs (subpopulations of Pax7⁺MyoD⁻, Pax7⁺MyoD⁺, and Pax7⁻MyoD⁺) were calculated. Previous research reported that Pax7 indicates quiescent subpopulations of SCs and MyoD is a marker for SCs activation and differentiation (Liu et al., 2012). Most cells in all experimental conditions were MyoD⁺, indicating that they were committed to a myogenic fate. A very low percentage (< 1.0%) of cells were quiescent (Pax7⁺MyoD⁻). The percentage of MyoD⁺ cells was not significantly altered by hypoxia or rescue. Hypoxia significantly increased the percentage of Pax7⁺ SCs over normoxia (from 66.3% to 77.1%). Virtually all these cells were also MyoD⁺ (65.7% and 77.0%, respectively), characteristic of SCs that are considered to be in a proliferative state but committed to myogenesis. The percentage of Pax7⁺/MyoD⁺ cells in the rescue group did not differ significantly from normoxic controls. Conversely, hypoxia significantly decreased the percentage of Pax7⁻/MyoD⁺ cells (31.8%, normoxia vs. 20.6%, hypoxia) which began the process of myogenic differentiation. In the rescue group, the abundance of this subpopulation was not statistically distinguishable from either normoxia or hypoxia. These data suggest that hypoxia exposure promotes the proliferation of activated SCs by maintaining Pax7 expression and suppresses myogenic potential of SCs.

Hypoxia inhibits myogenic potential of satellite cells

The effects of hypoxia on myogenic differentiation were further explored by inducing SCs to undergo myogenesis under each of the three treatment conditions (Figure 13A-F). Hypoxia (48 h) significantly decreased fusion of myotubes into larger fibers, based on the number of nuclei per myofiber (Figure 13G). In addition, hypoxia significantly decreased the percentage of multinucleate fibers (fusion index) vs. normoxia (Figure 13H). In the rescue group, fusion index was intermediate between both normoxic and hypoxic cultures, indicating a partial rescue of fusion when cells were returned to normoxia.

Hypoxia induces reactive oxygen species production

Both metabolic activity and reactive oxygen species have been shown to influence the myogenic program of SCs. The effects of hypoxia on mitochondrial metabolism were

measured using XTT assay, which reflects the activity of mitochondrial dehydrogenases in live cells. As shown in Figure 14A, hypoxia significantly decreased mitochondrial activity vs. normoxia. This effect was partially rescued by a return to normoxia, with the rescue group significantly different from both normoxia and hypoxia conditions. In contrast, hypoxia significantly increased intracellular ROS production, based on assay of DCFDA (Figure 14B). Increases were mitigated in the rescue group, and interestingly were significantly lower than in normoxic controls.

Hypoxia increases accumulation of lipid droplets in myotubes

The metabolic adaptation to hypoxia has been shown to result in formation of lipid droplets in multiple cell types. To investigate the effects of hypoxia on lipid ac within mature myotubes, myogenesis was induced for five days under normoxic conditions, after which cells were exposed to hypoxia for 48 h in differentiation media supplemented with fatty acids. Bodipy staining of neutral lipids revealed that small intramyocellular lipid droplets accumulated within myotubes after fatty acid supplementation, and this response was significantly increased under hypoxia (Figure 15).

Changes in protein expression involved in myogenesis, energy metabolism, and cellular stress

Western blotting was used to evaluate the effects of hypoxia on proteins involved in proliferation, myogenesis, energy metabolism, cellular stress (Figure 16). Expression of myogenin increased over the 48 h of culture, reflecting the progression of myogenic differentiation under normoxia. Interestingly, myogenin was barely detectable in SCs cultured under hypoxia conditions. Akt expression was increased by hypoxia exposure. Additionally, hypoxia activated the energy-sensing kinase AMPK. The cell stress-associated kinases P38MAPK and NF- κ B were also activated by hypoxia. Most changes in protein expression or phosphorylation after 48 h were almost not rescued by reoxygenation.

Hypoxia influences transcriptomic profiling in differentiating satellite cells

Targeted RNAseq was used to identify how hypoxia interacts with the myogenic program in SCs. Differentially expressed genes were investigated between differentiating SCs cultured under normoxia or hypoxia for 48 h hypoxia exposure. The PLS-DA analysis revealed a distinct separation between normoxia and hypoxia throughout myogenic differentiation (Figure 17). Expression levels of 126 of the 522 genes represented on the

panel were significantly affected by hypoxia (FDR $p \leq 0.05$), including 58 genes upregulated and 68 genes downregulated. Patterns of changes in expression are visualized in the heatmap (Figure 18). A total of 26, 54, 45, and 97 genes differed significantly after 12, 24, 36, and 48 h hypoxia exposure, respectively (FDR $p < 0.05$), reflecting changes that occur after hypoxia exposure. In the four-time sets, 13 genes showed significant differences in the four-time sets. Of these, genes upregulated by hypoxia include cochlin, extracellular fatty acid-binding protein (EXFABP), hexokinase 2 (HK2), myosin heavy chain 1E (MYH1E), parvalbumin (PVALB), and retinol binding protein 4 (RBP4). Those expressed at lower levels in hypoxia include myostatin, glycerol-3-phosphate dehydrogenase 1 (GPD1L2), insulin like growth factor 2 (IGF2), malate dehydrogenase 2 (MDH2), tollid-like protein 1 (TLL1), tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), and Wnt family member 5A (Wnt5A).

Discussion

Broiler breast muscle myopathies arise from aberrant repair of myofiber degradation that begins soon after hatch due to consequences of extremely rapid muscle growth. Localized tissue hypoxia is implicated as both a cause of the initial myofiber injury and a disruptor of the repair process (Sihvo et al., 2014; Velleman, 2019). Repair depends upon the behavior of SCs, but it is not well-understood how the hypoxic milieu within broiler breast muscle impacts their capacity to proliferate, differentiate, and contribute to formation of muscle fibers. We found that acute hypoxia favors myoblast proliferation while suppressing entry of cells into the differentiation pathway and impeding myocyte fusion and fiber growth. Myotube formation was not completely inhibited, but those that formed after 48 h were smaller in diameter and contained fewer nuclei. These effects were paralleled by decreased abundance of myogenin protein, the myogenic regulatory factors (MRF) responsible for inducing myocyte fusion and myotube growth, and by activation of signaling pathways and expression of genes associated with cellular stress.

Hypoxia in skeletal muscle often results from tissue injury that damages the vasculature or creates inflammation that limits perfusion, and therefore coincides with the need for muscle repair. Numerous studies (including ours) from multiple species have shown that hypoxia activates satellite cell proliferation but suppresses myogenic differentiation and impedes myotube growth. Based on this interrelationship, hypoxia has been proposed to act as a checkpoint that synchronizes myofiber repair with oxygen availability (Beaudry et al., 2016; Chaillou and Lanner, 2016). According to this concept, hypoxia primes muscle tissue

for repair by activating satellite cell proliferation but suppressing terminal differentiation and myotube maturation until adequate perfusion is restored by angiogenesis or resolution of inflammation. In this context, hypoxia is a physiologically beneficial and transient stimulus that insures healthy repair of damaged myofibers, after which SCs rapidly return to their quiescent state. In broilers, however, hypoxia within the breast muscle is thought to be consistent and possibly progressive as the breast muscle rapidly hypertrophies during the progression to market age. Hypoxia and myofiber degradation are thought to begin in the first two weeks post-hatch in broilers that develop lesions, followed by repeated cycles of fiber repair and degradation to market age. Therefore, the role of hypoxia as a transient, homeostatic signal that regulates both proliferation and differentiation of SCs may be disrupted in ways that contribute to formation of breast muscle lesions (Papah et al., 2017). For example, as a stem cell, SCs are thought to have a finite capacity for cell division. Persistent stimulation of proliferation by hypoxia may exhaust the ability of SCs to both replenish themselves and repair the myofiber damage that begins in the first few weeks after hatch (Meloche et al., 2018). Transcription of Pax7, which is sufficient to maintain SCs proliferation, is a direct target of hypoxia through both a hypoxia response element in the gene's promoter (Sinha et al., 2019) and hypoxia-regulated miRNAs (miR-1 and miR-206) that control Pax7 expression (Chen et al., 2010; Liu et al., 2012). Consistent with the concept of stem cell exhaustion, the proliferative capacity of SCs has been shown to decline with both age and fiber hypertrophy in broilers raised to market age (Daughtry et al., 2017). The extent of proliferation that is required to deplete SCs renewal capacity in broilers is unknown. However, exhaustion of SCs caused by futile cell divisions has been associated with compromised repair and infiltration of fibrotic tissue in Duchenne's muscular dystrophy, a disease with histological similarities to myopathies in broiler breast muscle (Sacco et al., 2010).

Myogenic differentiation results from carefully orchestrated and sequential changes in the abundance of MRF through a combination of transcriptional and post-transcriptional mechanisms. MRF proteins have relatively short half-lives, allowing their stability to provide a nimble mechanism through which to regulate myogenesis in response to changing physiological cues. Ubiquitin-mediated degradation contributes a key aspect of spatiotemporal control to myogenesis by acting as on/off switch that degrades MRF and controls transition between stages of differentiation. We found that myogenin protein abundance, but not mRNA level, was significantly decreased by hypoxia, suggesting that hypoxia enhances degradation of myogenin protein. Diminished myogenin levels are

consistent with our observations that hypoxia significantly reduced myocyte fusion, resulting in the formation of smaller myofibers. Therefore, these results indicate that hypoxia can impair myogenesis in broiler SCs by reducing the stability of myogenin and potentially other MRF. This concept is consistent with a previous study using myoblasts, in which hypoxia was shown to inhibit myogenesis by destabilizing the protein MyoD. Although we did not assess the abundance of MyoD using western blotting, we did observe a decrease in the relative abundance of MyoD⁺ SCs using immunocytochemistry. Therefore, MyoD protein stability may have also been affected by hypoxia, as was reported for C2C12 cells. It is also interesting to note that hypoxia significantly increased expression of basic helix-loop-helix family member e40 (BHLHe40), which has been shown to mediate hypoxia-induced repression of myogenin expression and myogenesis in mice. BHLHe40 represses myogenin transcription by disrupting formation of the P/CAF-MyoD complex that is required for transcription. Overexpression of BHLHe40 was shown to inhibit myogenesis in mouse myoblasts, whereas knockdown induced precocious differentiation (Wang et al., 2015). Although we did not find effects of hypoxia on myogenin mRNA, these findings point to a potential role for BHLHe40 as a mediator of hypoxia effects in broiler myoblasts (Di Carlo et al., 2004).

Cells metabolically adapt to hypoxia by shifting metabolism from oxidative phosphorylation to glycolysis, as a means to maintain ATP production when oxygen supply is limited. Accordingly, genes encoding key enzymes in glycolysis, and many glucose transporters, contain hypoxia response elements in their promoter regions, allowing rapid upregulation when HIF-1 α protein is stabilized by a decline in intracellular oxygen tension. Increased expression of hexokinase (HK; at all time points) and SLC2A14 (glucose transporter 14; at 24 h) in our study, along with stabilization of HIF-1 α protein, reflect this adaptive response to hypoxia. Although metabolic reprogramming allows cells to sustain ATP production under low oxygen, the transition in substrate utilization imposes significant metabolic stress on cells due to both increased production of ROS and diminished cellular energy. Consequently, we found that acute hypoxia significantly increased ROS levels and activated signaling through AMPK. Production of ROS increases under acute hypoxia as a result of inefficient electron transport and leakage of electrons from the electron transport chain (Zuo and Clanton, 2005). Evidence of increased ROS and oxidative stress have been reported to occur as early as two weeks posthatch and to precede the development of wooden breast and other myopathies (Papah et al., 2017). AMPK adapts cellular metabolism to low energy states by upregulating catabolic pathways and suppressing those that are

energetically costly. Under hypoxia, AMPK promotes cell survival and a continued supply of ATP by enhancing glucose uptake and glycolysis (Mu et al., 2001). Activation of AMPK involves phosphorylation on Thr172 and canonically results from an increase in the cellular AMP/ATP ratio. Increased cellular ROS can also activate AMPK signaling, which may have contributed to the increased AMPK phosphorylation shown in our Western blot results.

We found that the challenge of survival under low oxygen, low cellular energy, and increased ROS activated stress and inflammatory signaling pathways in broiler myoblasts. The NF- κ B signaling pathway orchestrates inflammatory and immune responses to a wide array of cellular stressors (Liu et al., 2017). In skeletal muscle, NF- κ B has been shown to augment metabolic adaptations to hypoxia, in addition to its role in mediating inflammation (Remels et al., 2015). Hypoxia also markedly (up to $\sim$ 8-fold) induced expression of CCL20, a downstream target of NF- κ B and HIF-1 α . Expression of this chemokine, which recruits Th17 and dendritic cells to sites of inflammation, was upregulated compared to normoxia at all time points. Notably, CCL20 has been implicated in the development of inflammatory myositis in humans (Beringer et al., 2018). P38MAPK, like NF- κ B, communicates a variety of extracellular stressors to adaptive response pathways in the cytoplasm and nucleus (Beringer et al., 2018). Hypoxia has been shown to rapidly induce phosphorylation of P38MAPK- α (Haddad, 2004), consistent with its activation in our model. Activation of each of these stress-mediating pathways paralleled stabilization of HIF-1 α protein, in those levels after returning cells to normoxia for 24 h, after 24 h under hypoxia, were comparable to those of normoxic controls. Therefore, effects on hypoxia on stress signaling appear to be rapidly reversible by restoration of O₂ levels. In broilers, however, persistent hypoxia may cause sustained activation of these, which could contribute to the inflammation and disrupted myofiber repair that are associated with breast muscle myopathies. For example, although physiological levels of NF- κ B activity support muscle repair, constitutive activation of NF- κ B signaling impairs repair and can promote fibrosis in place of healthy muscle tissue (Mourkioti et al., 2006; He et al., 2013; Ogura et al., 2015; Straughn et al., 2019).

Exogenous stressors, including hypoxia, induce many cell types to form cytoplasmic lipid droplets, in which fatty acids are sequestered as neutral triglyceride (Jarc and Petan, 2020; Yokota et al., 2021). Lipid droplets buffer cells under metabolic stress from the potential cytotoxic effects of free fatty acids and sequester energy during periods of deprivation. We found that hypoxia induced the formation of cytoplasmic lipid droplets in mature myotubes. Few and small droplets were present under normoxia in some cells, with

both number and size increased by hypoxia. Lipid accumulation in lipid droplets during hypoxia results primarily from the uptake of free fatty acids via fatty acid binding proteins (FABPs). Our targeted RNAseq data indicated that expression of FABP2 was significantly increased by hypoxia, while expression of the lipases monoglyceride lipase (MGLL) and adipose triglyceride lipase (PNPLA2) was downregulated, all of which may have contributed to droplet expansion. While lipid droplet formation is cytoprotective under hypoxia, it could also contribute to the ectopic lipid accumulation that has been linked to the etiology of breast muscle myopathies in broilers. We did not measure the effects of hypoxia on lipid accumulation in SCs prior to myotube formation, although we expect that lipid droplets would also have formed, given that this is a response to hypoxia in multiple cell types. It is worth noting that very few of the genes that were upregulated in SCs after 48 h of hypoxia are also upregulated in adipogenesis of broiler chick preadipocytes, using the same targeted RNAseq panel (data not shown).

Our targeted RNAseq panel is limited in scope in comparison to whole genome approaches. Nonetheless, we identified genes of interest that, to our knowledge, have not previously been associated with consequences of hypoxia in broiler SCs. Expression of parvalbumin (PVALB), which plays a critical role in buffering intracellular Ca^{2+} in fast-twitch muscle fibers, was upregulated by hypoxia at all time points in our study. Although there are no studies on the link between hypoxia and PVALB in skeletal muscle, various cell types (including cardiomyocytes) show increases in cytosolic calcium under hypoxia (Berna et al., 2001; Toescu, 2004). Increased intracellular Ca^{2+} has been implicated as a causative factor in wooden breast. Notably, PVALB expression is elevated in breast muscle of birds affected with this myopathy (Mutryn et al., 2015; Lake et al., 2021). EXFABP is a stress protein that is not only physiologically necessary at the developmental stage but also appears in tissues due to oxidative stress under pathological conditions (Descalzi Cancedda et al., 2001). In chicken embryonic fibroblasts, EXFABP is markedly stimulated by hypoxia-induced growth arrest, alluding that hypoxia is linked to the control of this gene expression and proliferation (Erb et al., 2016). Interestingly, EXFABP is increased in the pectoralis major of both affected and high-feed efficient broilers (Papah et al., 2018; Abasht et al., 2019). Cochlin is primarily known as the major non-collagen component of the extracellular matrix in inner ear (Robertson et al., 1998), but our results demonstrate that it is markedly and persistently upregulated by hypoxia in SCs. Although its function has not yet been studied in SCs and skeletal muscles, cochlin has been shown to play a role in maintaining self-renewal of embryonic stem cells as a target gene of BMP4 (Zhang et al., 2013). Malate

dehydrogenase 2, a key enzyme of the TCA cycle, catalyzes the reversible oxidation of malate to oxaloacetate. Several studies show that hypoxia exposure decreased MDH2 activity, which is consistent with our results (Horscroft and Murray, 2014; Capitanio et al., 2017). This gene has been identified as a biomarker for Duchenne muscular dystrophy (Signorelli et al., 2020), and wooden breast also shows reduced MDH2 expression, along with increased levels of malate (Abasht et al., 2016; Pampouille et al., 2019).

Collectively, our results support the concept that cellular hypoxia disrupts the pathways that contribute to muscle repair in broilers. We have identified several novel effects of hypoxia on the early steps of myogenic differentiation and repair that provide further insight into the potential basis for breast muscle myopathies in broilers. However, limitations of these studies should be noted. In particular, we modeled normoxia using oxygen levels that are standard in *in vitro* studies but that do not accurately reflect the oxygen tension in muscle *in vivo*. We also used a model of acute hypoxia and recognized that cells *in vivo* may adapt to chronic hypoxia in ways that overcome the effects shown in our work. Finally, our targeted RNAseq panel revealed an array of differentially expressed genes of interest but did not capture the full suite of the avian transcriptome. It is important to point out that, in this study, reoxygenation rapidly reversed the majority of hypoxia's effects of SCs. These effects suggest that measures to improve tissue perfusion within the pectoralis major muscle hold promise for preventing the cycle of damage and compromised repair that underlie the formation of breast muscle lesions. Future studies will address such strategies.

Appendix 4

Table 4. List of antibodies used for western blotting

Antibodies	Source	Cat. No.	MW (kDa)	Host	Dilution
AKT	Cell Signaling Technology	4685	60	Rabbit	1/500
AMPK	Cell Signaling Technology	5832	62	Rabbit	1/500
HIF-1 α	EMD Millipore	7628	78	Rabbit	1/200
NF-kB p65	Santa Cruz Biotechnology	sc-372	65	Rabbit	1/500
P38	Santa Cruz Biotechnology	sc-7972	42	Mouse	1/500
Phospho-AKT (S473)	Cell Signaling Technology	3527	60	Rabbit	1/500
Phospho-AMPK (T172)	Cell Signaling Technology	2535	62	Rabbit	1/200
Phospho-P38 (T180/Y182)	Cell Signaling Technology	4511	43	Mouse	1/200
Phosphp NF-kB p65 (S536)	Santa Cruz Biotechnology	sc-33020	65	Rabbit	1/200
β -actin	Santa Cruz Biotechnology	sc-47778	44	Mouse	1/500

Table 5. Differentially expressed genes (FDR < 0.05) in normoxia versus hypoxia at all time points

Gene ¹	Gene Identifier	Fold Change (hypoxia vs normoxia)			
		12 h	24 h	36 h	48 h
COCH	ENSGALT00000016132	5.41	7.96	6.47	6.33
EXFABP	ENSGALT00000069086	13.65	10.70	4.06	3.99
MSTN	ENSGALT00000066640	0.22	0.17	0.21	0.16
GPD1L2	ENSGALT00000018718	0.27	0.20	0.22	0.16
HK2	ENSGALT00000060341	1.96	2.37	2.02	4.88
IGF2	ENSGALT00000053800	0.47	0.28	0.36	0.45
MDH2	ENSGALT00000003016	0.63	0.58	0.60	0.56
MYH1E	ENSGALT00000070926	1.61	2.40	2.15	2.50
PVALB	ENSGALT00000020453	3.14	3.09	1.96	2.05
RBP4A	ENSGALT00000061509	1.82	1.82	1.60	1.79
TLL1	ENSGALT00000015583	0.59	0.56	0.50	0.48
TNFRSF1B	ENSGALT00000061967	0.19	0.33	0.26	0.22
WNT5A	ENSGALT00000070747	0.66	0.55	0.62	0.74

¹ COCH, cochlin; EXFABP, extracellular fatty acid-binding protein; MSTN, myostatin; GPD1L2, glycerol-3-phosphate dehydrogenase 1; HK2, hexokinase 2; IGF2, insulin like growth factor 2; MDH2, malate dehydrogenase 2; MYH1E, myosin, heavy chain 1E; PVALB parvalbumin; RBP4A, retinol binding protein 4A; TLL1, tollid-like protein 1; TNFRSF1B, tumor necrosis factor receptor superfamily member 1B; WNT5A, Wnt family member 5A.

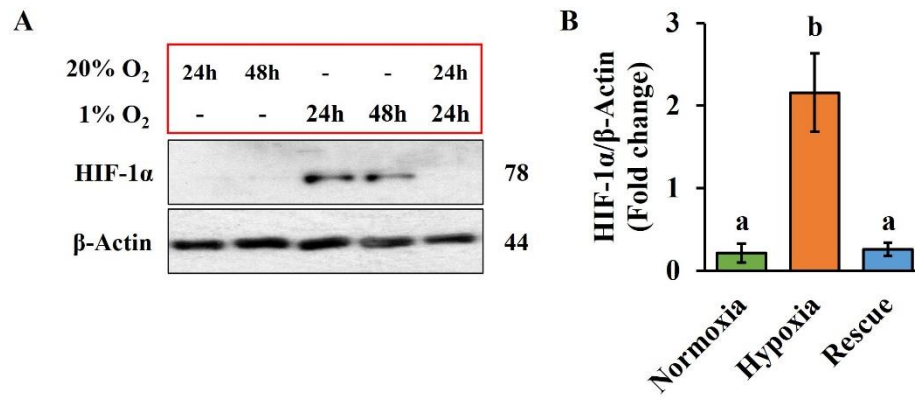


Figure 10. Hypoxia stabilizes HIF-1 α in broiler satellite cells

Proteins were extracted from 4 biological replicates/treatment. β -actin was used as a loading control for western blotting. Stabilization of HIF-1 α confirms effects of hypoxic conditions. Band intensity at 48 h was measured with densitometric analysis and normalized to the intensity of β -actin. The data were expressed as the mean $\pm$ SD. $n = 4$. ^a and ^b $P < 0.05$.

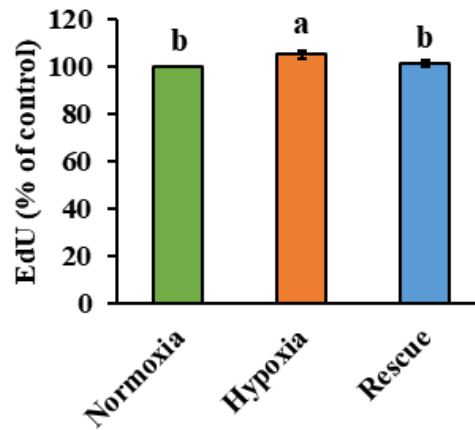


Figure 11. Effects of 48 h hypoxia or rescue on the proliferation of broiler satellite cells

EdU proliferation assay was performed to evaluate DNA replication and its values were expressed as a percent of control. The data were expressed as the mean $\pm$ SD. $n = 10$. ^{a,b,} and ^c $P < 0.05$.

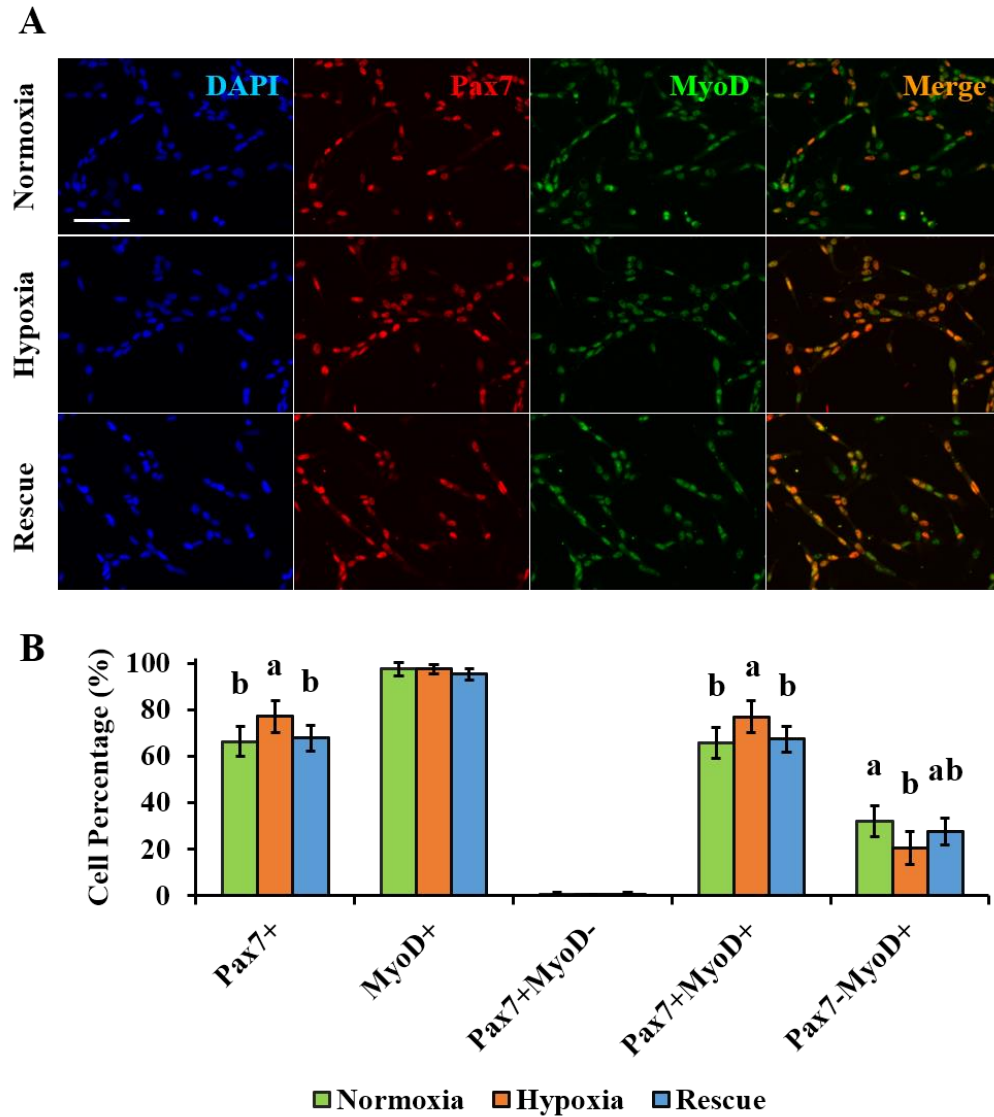


Figure 12. Effects of 48 h hypoxia or rescue on heterogeneity of broiler satellite cells

(A) Representative images of broiler satellite cells cultured in growth media under 21% O₂ (normoxia) or 1% O₂ (hypoxia) for 48 h and immunostained with Pax7 (red) and MyoD (green). Nuclei were counterstained with Hoechst33342 (blue). Scale bar = 150 μ m (B) Quantification of the quiescent (Pax7⁺MyoD⁻), proliferating (Pax7⁺MyoD⁺), and differentiating (Pax7⁻MyoD⁺) satellite cells. The number of nuclei was counted by ImageJ. The data were expressed as the mean $\pm$ SD. $n = 10$. ^a and ^b $P < 0.05$.

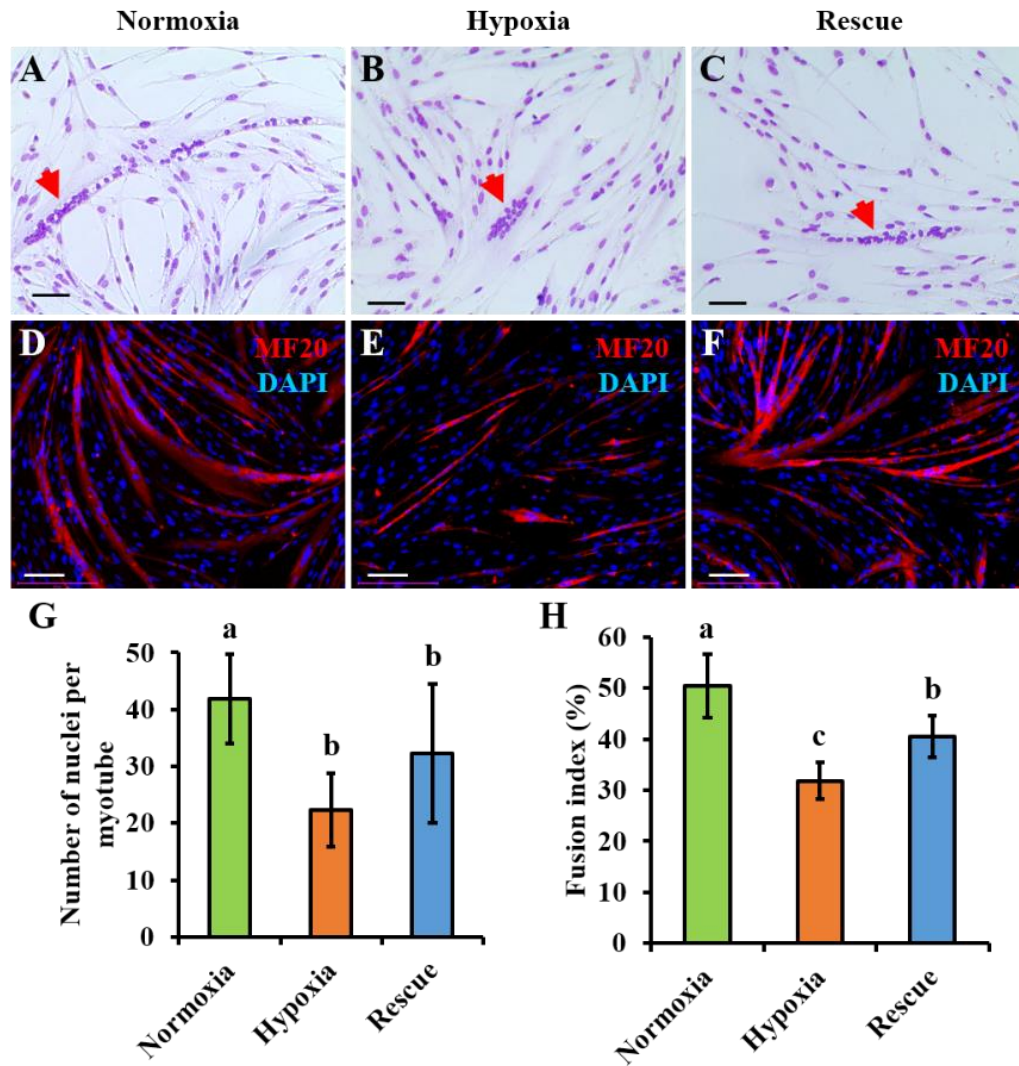


Figure 13. Effects of 48 h hypoxia or rescue on myogenic differentiation of broiler satellite cells

After being plated, satellite cells were cultured in growth media under normoxia for 24 h. The cells were then transferred to a hypoxia chamber, where they were cultured for an additional 48 h in differentiation media (A-C) HEMA 3-stained myotubes ($n = 10$). Red arrows indicate fused myonuclei. Scale bars = 50 μm (D-F) Immunostained myotubes ($n = 3$) against MF 20 (myosin, red) and Hoechst33342 (nuclei, blue). Scale bars = 150 μm (G) number of nuclei per myotube (H) fusion index (%). The data were expressed as the mean $\pm$ SD. ^a and ^b $P < 0.05$.

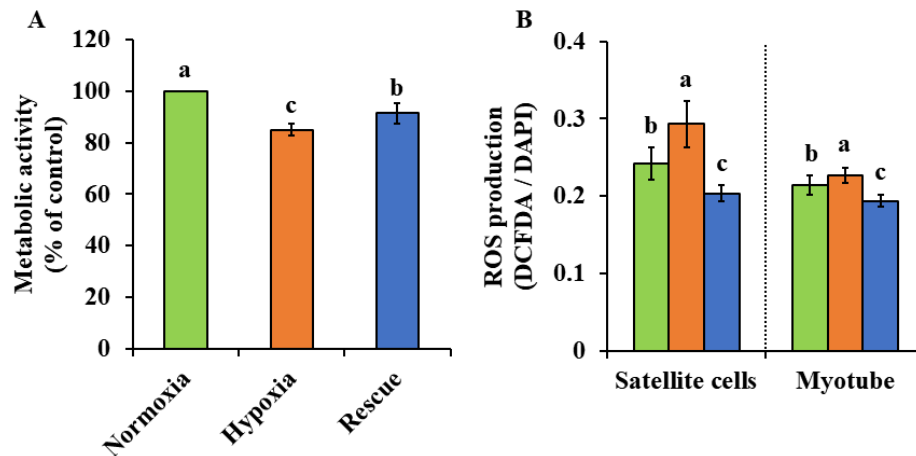


Figure 14. Effects of 48 h hypoxia or rescue on reactive oxygen species production in broiler satellite cells and myotubes

Satellite cells were plated and cultured in growth media under normoxia for 24 h. After this, the cells were transferred to a hypoxia chamber and cultured for an additional 48 h in growth media. Satellite cells were induced to form myotube in differentiation media for 3 d. After myotube formation, the myotube culture was exposed to hypoxia in differentiation media for 48 h (A) XTT assay was performed to evaluate proliferation of satellite cells based on metabolic activity ($n = 10$) (B) reactive oxygen species was measured by DCFDA assay ($n = 5$). The data were expressed as the mean $\pm$ SD. ^a, ^b, and ^c $P < 0.05$.

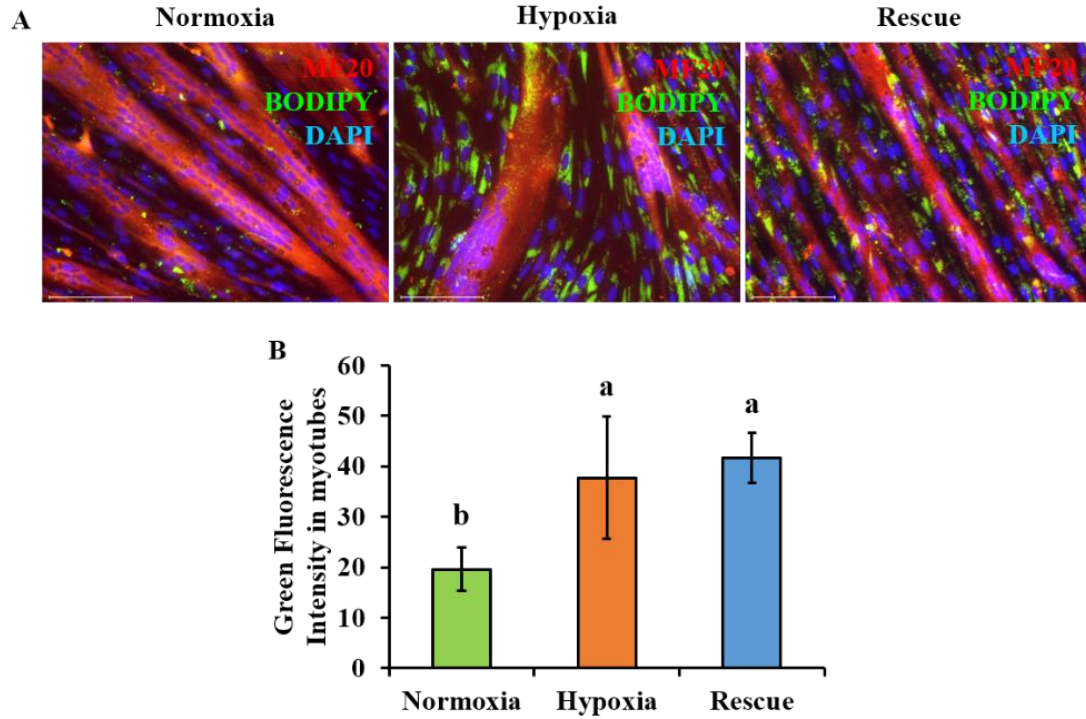


Figure 15. Effects of 48 h hypoxia or rescue on intramyocellular lipid accumulation in mature myotubes

(A) immunofluorescent staining of myh1-positive mature myotubes (red) with BODIPY (493/503), to visualize lipid droplets (green), and Hoechst33342 (blue) to visualize nuclei. Lipid accumulation within myotubes was induced with the differentiation media supplemented with 94 ng/mL linoleic acid and 94 ng/mL oleic acid. Scale bars = 75 μ m (B) quantification of lipid accumulation (green fluorescence intensity) in myotubes. The data were expressed as the mean $\pm$ SD. n = 10. ^a and ^b $P < 0.05$.

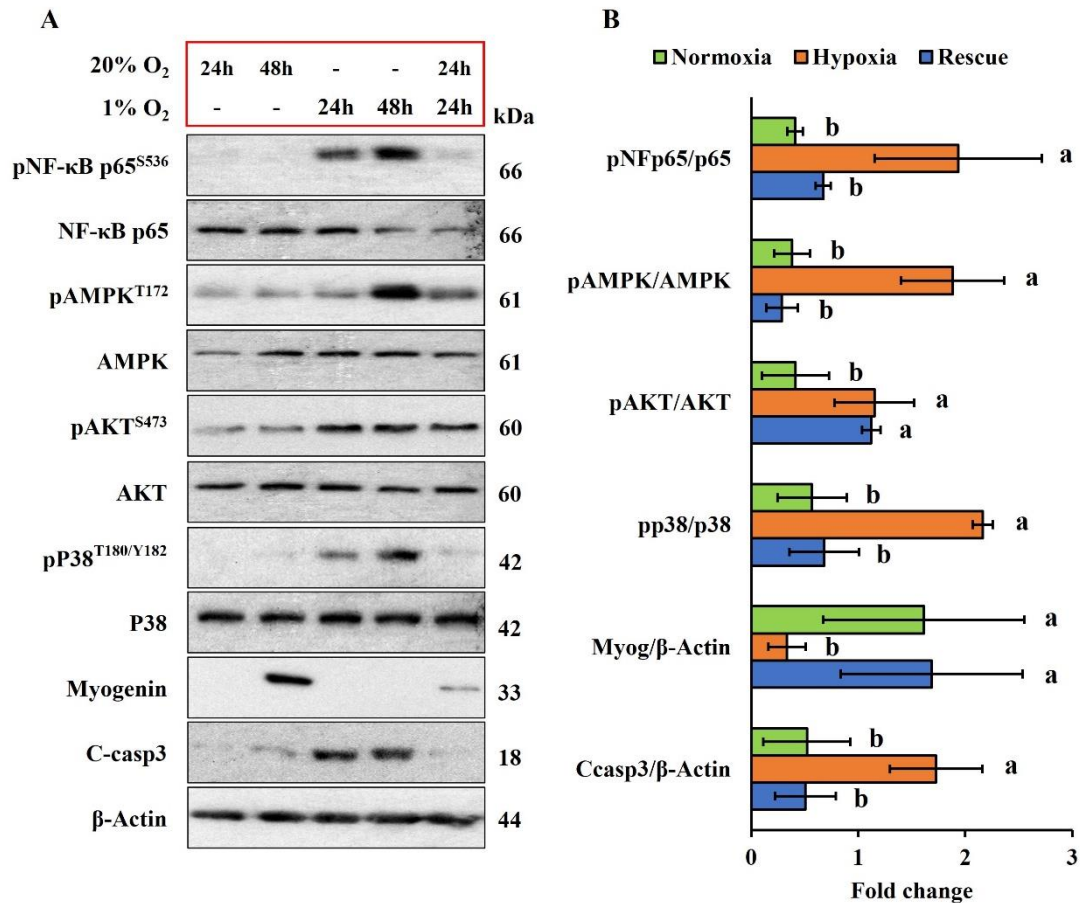


Figure 16. Effects of hypoxia (24 or 48 h) or rescue on metabolic signaling in broiler satellite cells

(A) western blotting showing the protein expression of NF-κB, AMPK, AKT, P38, and myogenin. Proteins were extracted from 4 biological replicates/treatment. β-actin was used as a loading control for the western blotting (B) Band intensity at 48 h was measured with densitometric analysis and normalized to the intensity of non-phosphorylated form or β-actin. The data were expressed as the mean ± SD. n = 4. ^a and ^b $P < 0.05$.

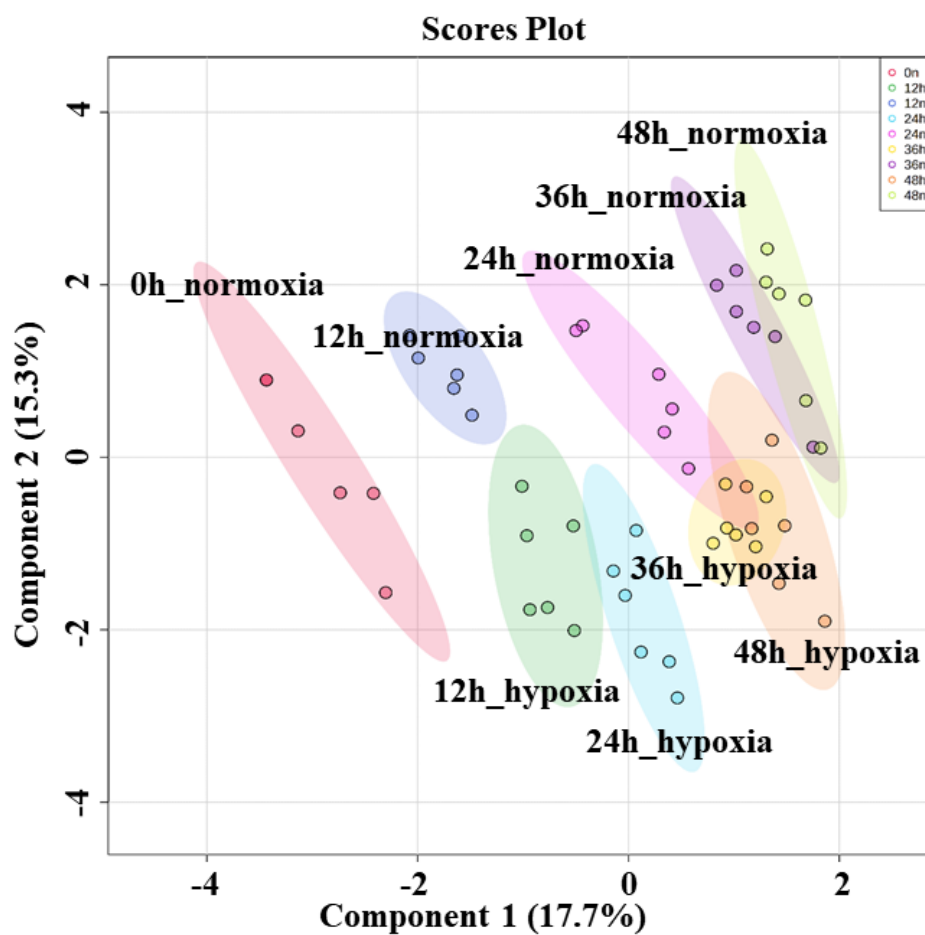


Figure 17. PLS-DA visualization of sample clustering in satellite cells across differentiation under normoxia and hypoxia

Data from each sample is encoded by color. Analyses were performed using Metaboanalyst (metaboanalyst.ca) using default settings. Statistical significance of the sample clustering was evaluated with a permutation test (1,000 times) ($P < 0.001$).

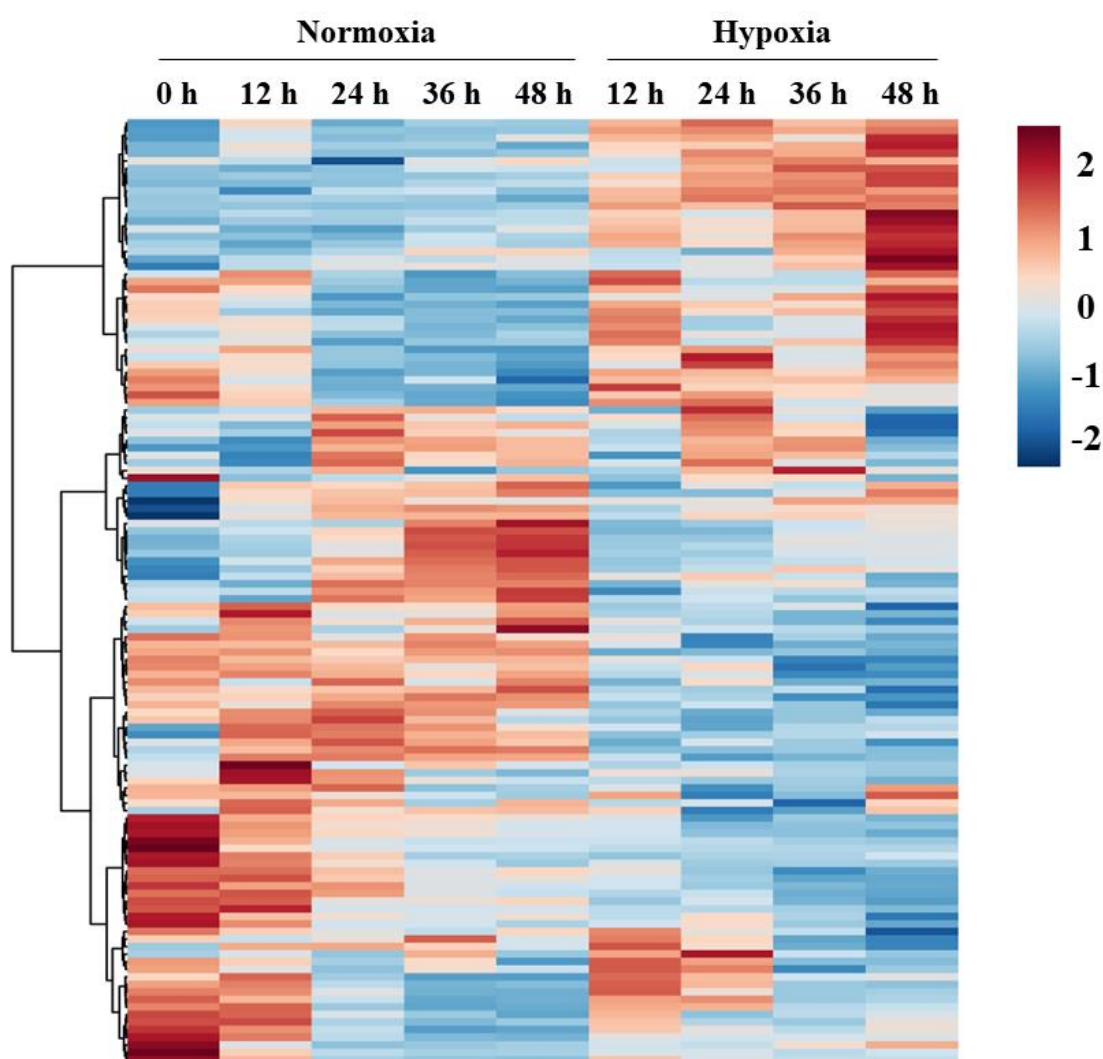


Figure 18. Heatmap of 125 differentially expressed genes by hypoxia in differentiating broiler satellite cells

A total of 26, 53, 44, and 97 genes differed significantly after 12, 24, 36, and 48 h hypoxia exposure, respectively. Data were normalized by total counts and log transformed. Red indicates up-regulation, and blue indicates down-regulation. $n = 6$. FDR $P < 0.05$.

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